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13. ABSTRACT (Maximum 200 Words)

Ab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. My research project consists in the study of Rab GTPases, the way in which they regulate intracellular transport, and the elucidation of mechanisms by which proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation and cancer. Our laboratory has extensively characterized Yip1p, membrane proteins which appear to play a role in Rab-mediated membrane transport in *Saccharomyes cerevisiae*. The major accomplishment this final year was the elucidation of the importance of the prenylation on the activity and localization of Rab GTPases. The results will be discussed in terms of the relevance to treatment for human cancers.

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INTRODUCTION:

During the development and progression of human cancer, cells undergo numerous changes in morphology, proliferation and transcription. Some of the molecular mechanisms involve in these changes have involved the members of the Ras superfamily of small GTPases. Rab proteins constitute the largest group of this superfamily (1). They are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. It is becoming more apparent that Rab GTPases,by regulating intracellular transport and events such as regulated exocytosis (in mammary gland for example) play a crucial part in uncontrolled cellular proliferation and cancer. My research project aimed to examine the mechanisms by which Rab GTPases regulate intracellular transport, and the elucidation of the links connecting intracellular protein traffic to uncontrolled cellular proliferation and cancer. In my first year, I reported the extensive characterization of a Rab interacting membrane protein Yop1p, the yeast homolog of the familial adenomatous polyposis locus gene known as TB2(2). We reported that overexpresion of Yop1p resulted in the accumulation of internal vesicles and cell death. Furthermore, we showed that Yop1p complexes with Yip1p, a membrane protein in yeast essential for membrane transport. In my second year of funding, the focus of my research was on understanding the role of Yip1p and Yop1p in Rab function and to begin the characterization of the interactions of Rab proteins with the mammalian homologs of Yip1p. Also, I define a new family of proteins called the YIP1 family (3,4). I also noted in my last report the finding that the double prenylation of Rabs was essential for localization and function. These results underscore the importance of the prenyl lipid groups in for Rab protein function and point towards an interesting link toward cancer research since it has been generally accepted that geranylgeranylated proteins have an important role in cellular proliferation (5).

In this last year, I have graduated and have finished the studies of this research project and the results culminated in a peer-journal publication in the journal Molecular Biology of the Cell (6). The present report will highlight the progress and the culmination of the project funded by DOD Breast Cancer Pre-doctoral grant.

BODY:

Rab GTPases play a crucial role in uncontrolled cellular proliferation and cancer by regulating intracellular transport and events such as regulated exocytosis (in mammary and prostate glands for example) and endocytic events. Evidence for this comes from a collection of microarray experiments utilizing cancer cell lines and tumors (7). Therefore, the understanding of the regulation of Rab proteins is crucial for the elucidation and possibly therapeutic approaches to cancer. During the period supported by the DOD pre-doctoral grant, I have characterize and define a family of membrane proteins (YIP1 family) that appears to have an important role in Rab protein regulation. In this last year the following results were obtained:

a) ***Prenylation of Rab GTPases:*** The vast majority Rab proteins are post-translationally modified with two geranyl-geranyl lipid moieties that enable their stable association with membranes. In this year I completed the studies that demonstrated the specific lipid requirement for Rab protein localization and function. Substitution of different prenyl anchors on Rab GTPases does not lead to correct function. In the case of *YPT1* and *SEC4*, two essential Rab genes in *S. cerevisiae*, alternative lipid tails cannot support life when present as the sole source of *YPT1* and *SEC4*. Furthermore, my data suggest that double geranyl-geranyl groups are required for Rab proteins to correctly localize to their characteristic organelle membrane. I then demonstrate that Yip1p specifically binds the di-geranyl-geranylated Rab and does not interact with mono-prenylated Rab proteins. This is the first demonstration that the double prenylation modification of Rab proteins is an important feature in the function of this small GTPase family, and adds specific prenylation to the already known determinants of Rab localization. For these studies, please see the attached manuscript " Double prenylation is a requirement for Rab protein localization and function"

b) ***Insights into the function of YIP1 and related proteins:*** In this last year I have conducted cell biological, biochemical and genetic experiments that suggest that Yip1p is a factor whose function is antagonistic to Rab-GDI and that it may function as a Rab-GDI receptor.

Rab-GDI is a crucial protein in the cycle of Rab GTPases. It is able to form a soluble heterodimer with the prenylated Rab and therefore mediate the translocation of Rabs between the cytoplasm and membranes (8,9). Rab-GDI delivers Rab GTPases to the correct intracellular compartment and retrieves them from membranes for recycling. To date, three isoforms have been isolated in mammalian tissues (α,β and γ) and in *S. cerevisiae*, only a single essential gene has been identified: *GDI1* or *SEC19* (10). The biological importance of GDI is underscored by the fact that there is one Rab-GDI for 11 Rabs in *S. cerevisiae*, and 3 GDIs for over 60 Rabs in mammalian cells. The affinity of Rab-GDI for a Rab has been reported to be in the nanomolar range (11). Therefore, in order for the Rab to be released from Rab-GDI and attach to membranes, the activity

of a protein called GDF (GDI Displacement Factor), is needed (12). To date, the molecular identity of this protein has remained elusive.

Interaction of Yip1p with Rab GTPases and Rab GDI:

In the manuscript appended, we show two hybrid data suggesting that Yip1p can interact with the 11 Rab proteins in yeast in a non-specific manner. This interaction is dependent on C-teminal double prenylation (6). In order to corroborate the two hybrid data of Rab-YIP1 interactions, I tagged Yip1p, with an MBP tag, and Rab proteins with a GST tag. Amylose resin pulldowns were performed and the blots were probed for GST to detect the association of Rab proteins with all Yip1p. As shown in Figure 1, Yip1p associated with full length GST-Rabs tested, except for GST-Ypt1ΔC, a construct lacking the C-terminal cysteines and therefore unmodified. These interactions are very similar to the ones described for Rab-GDI and Rab proteins ((8) and Figure 2 where a similar experiment was perfomed). The difference lies in the fact that Rab-GDI will bind a single prenylated Rab and Yip1p will not (6). In the newly solved structure for Rab-GDI complexed to a geranyl-geranyl substrate (13) demonstrates that the lipid is buried in a hydrophobic pocket. In this study, the authors model the second geranyl geranyl lipid moetie. Their analysis shows that the second lipid is not buried as the first lipid, it is rather exposed in a second hydrophobic groove. The fact that Yip1p absolutely requires the two lipids to interact with the Rabs, offers the exciting possibility that it could acts as a specific receptor for doubly prenylated Rabs bound Rab-GDI. We therefore wanted to explore the possibility of Yip1p binding Rab-GDI. For these experiments, I created a strain that expresses GST alone or GST- Rab GDI (bovine GDI) fusion protein under the control of a regulatable $GAL_{1/10}$ promoter. Each of these strains contains a multi-copy plasmid expressing GFP-tagged Yip1p of GFP-tagged Yip1p soluble domain. Tween-20 detergent solubilized total lysates were produced from mid-log phase cells and GST or GST-GDI were isolated on glutathione agarose beads followed by SDS-PAGE and

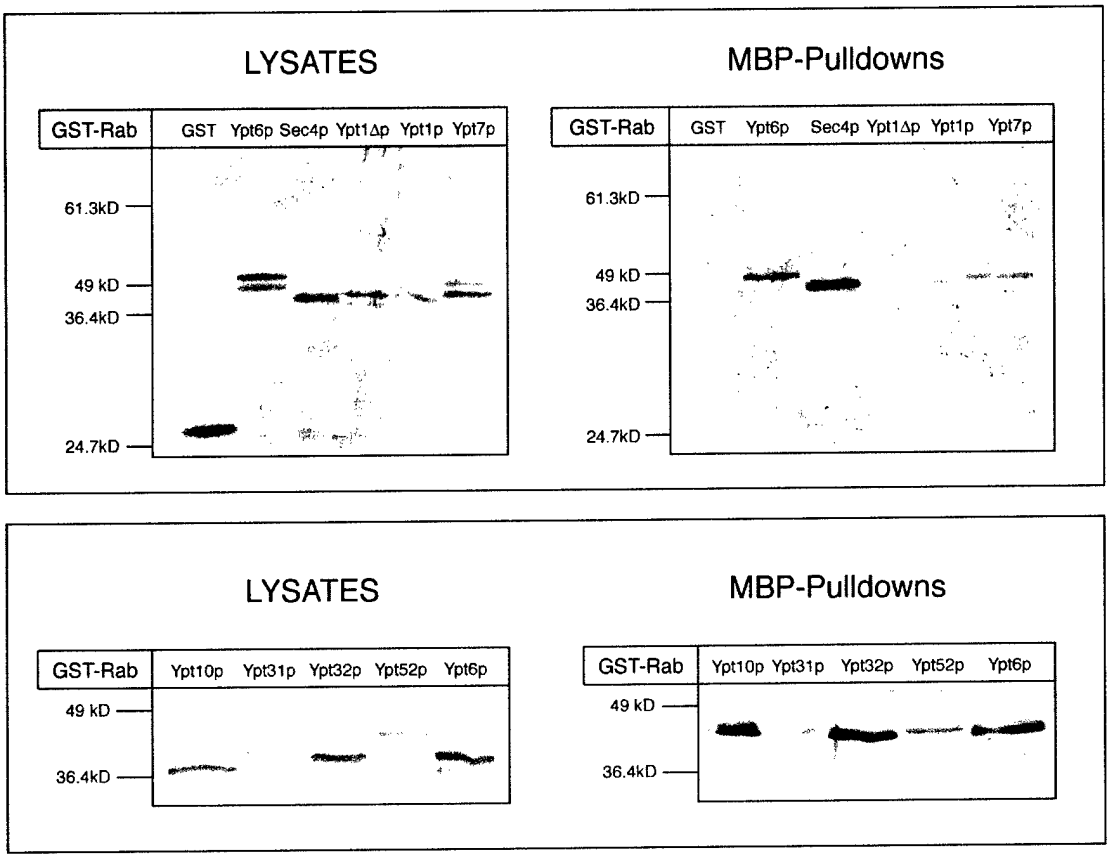


Figure 1. MBP-Yip1p interacts with various GST-Rabs in cellular lysates. Lysates were prepared from cells expressing both GST-tagged constructs and MBP-Yip1p. Detergent solubilizes lysates were incubated with amylose resin beads for 30 minutes at 4°C. After four washes, the bead-bound material was subject to SDS-PAGE analysis and Western Blotting. Membranes were probed with α-GST. All of the full length Rabs tested were detected after pulldowns except for Ypt1ΔC.

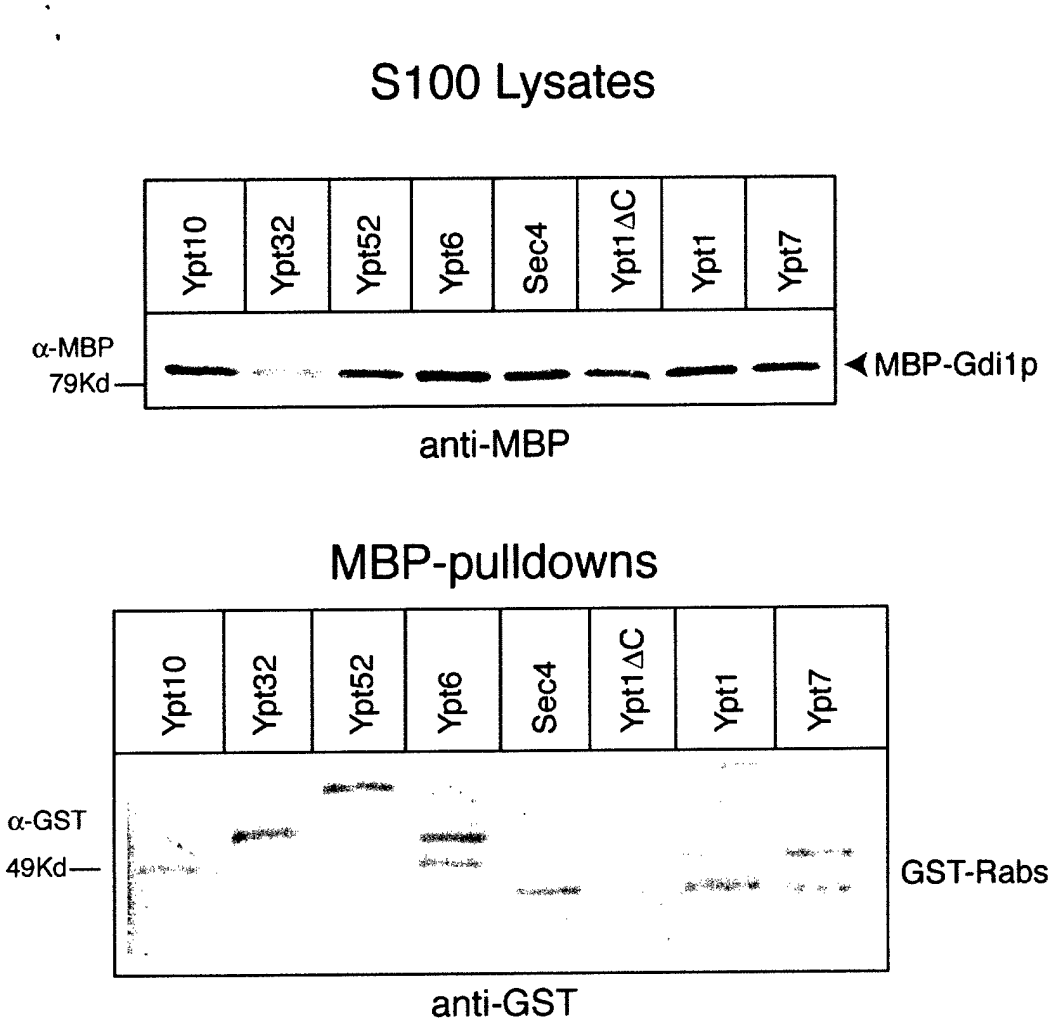


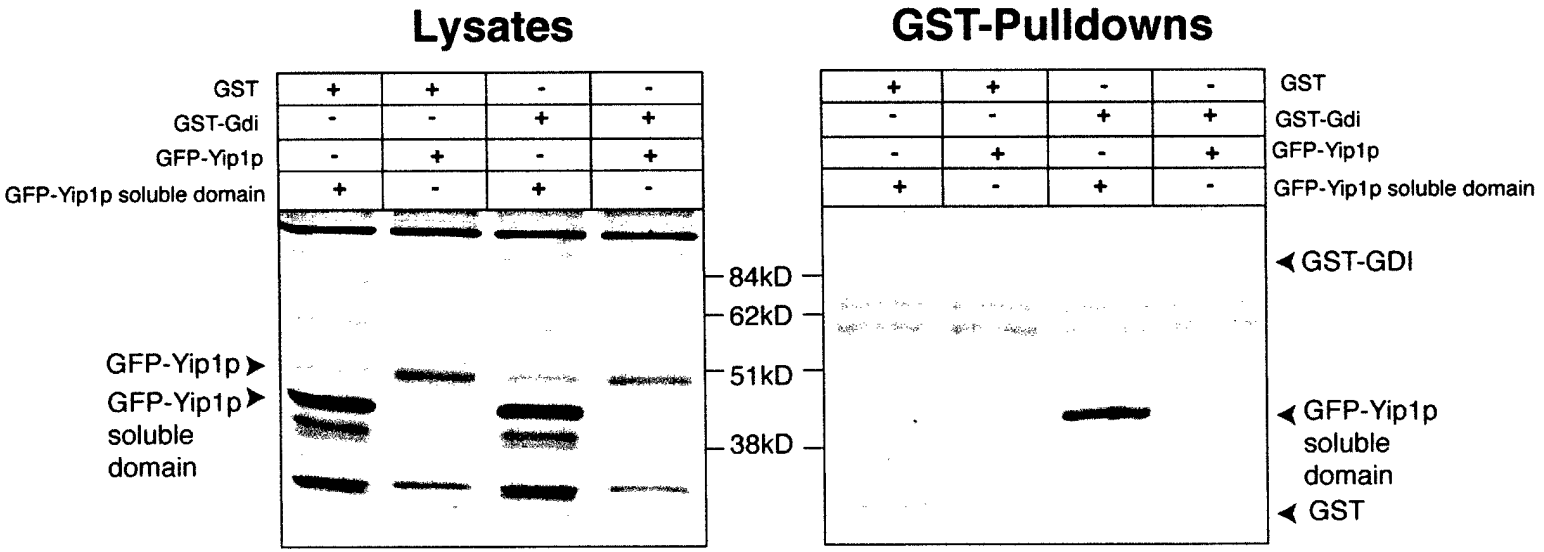
Figure 2. MBP-Gdi1p interacts with various GST-Rabs in cellular lysates. Lysates were prepared from cells expressing both GST-tagged constructs and MBP-Yip1p. Detergent solubilizes cytosolic lysates were incubated with amylose resin beads for 30 minutes at 4°C. After four washes, the bead-bound material was subject to SDS-PAGE analysis and Western Blotting. Membranes were probed with α-GST. All of the full length Rabs tested were detected after pulldowns except for Ypt1ΔC.

western bloting to detect the presence of GFP-Yip1p of GFP-Yip1p soluble domain using an α-GFP antibody. The results of these experiments are shown in Figure 3. GFP-soluble domain was detected in the GST-GDI pull-down but was not detected in the pull-down of GST alone. The interaction was not detected with the full length of Yip1p indicating the the C-terminus could be an inhibitory domain and that other proteins may be involved in this complex. However, one problem with these experiments is that the expression level of the full length of Yip1p is low compared with the soluble domain. This may have been the reason why we did not detect the interaction.

Functional relevance of Yip1p/Rab GDI interaction:

In order to try to understand and give some functional relevance to Yip1p /Rab-GDI interaction, we used two soluble domain mutants of Yip1p that render a physiological consequence and phenotype to yeast. One is the temperature sensitive allele *yip1-4* that was described in the manuscript appended (6). Cells bearing this allele have a restrictive temperature at 37°C. The second allele, is a dominant negative mutant (E76K or *yip1-6*), that when overexpressed, has a toxic effect on yeast (Figure 4). For these experiments, we cloned this mutant behind the Cu^{2+} promoter to regulate its expression. Cells plated on 0.5mM Cu^{2+} could grow fine (vector alone) and overexpression of wild type *YIP* or other mutations such as *yip1*^{D110A} (*yip1-11*) are not toxic. However, cells bearing *yip1-6* fail to grow in Cu^{2+} indicating that this mutation is dominant negative for growth (Figure 4).

Figure 3. Biochemical analysis of Rab-GDI and Yip1p interaction. Lysates were prepared from cells expressing both GST-tagged constructs and either GFP-Yip1p soluble domain or GFP-Yip1p. Membranes were probed with α-GFP. GFP-Yip1p soluble domain was detected after pulldowns with GST-Rab GDI but not with GST alone.


Lysates
GST-Pulldowns
GST
GST-Gdi
GFP-Yip1p
GFP-Yip1p soluble domain
84kD
62kD
51kD
38kD
GFP-Yip1p
GFP-Yip1p soluble domain
GST-GDI
GFP-Yip1p soluble domain
GST

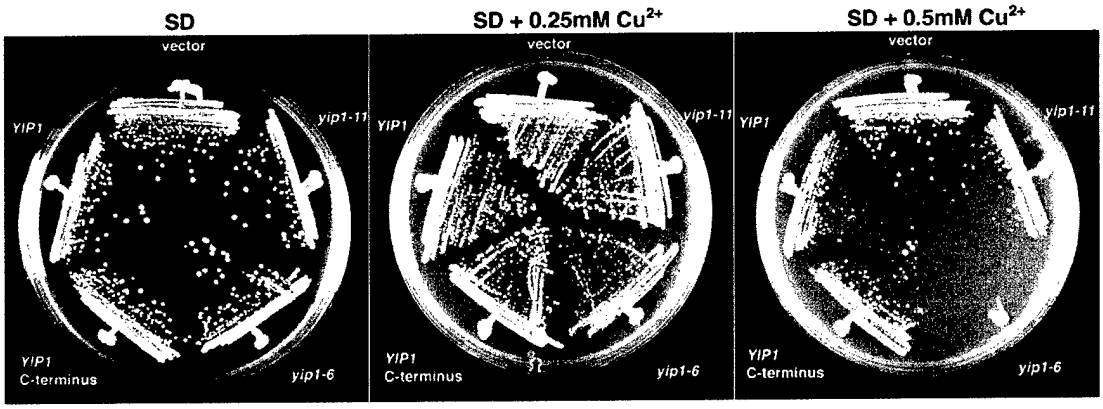


Figure 4 *yip1-6* is a dominant negative allele of *YIP1*.

A mutation in the soluble domain E76K of Yip1p leads to a dominant negative phenotype. Constructs were cloned under the control of the Copper promoter. These constructs were transformed into yeast and transformants were streaked in media containing Cu^{2+}.

We therefore investigated if the proteins encoded by these alleles *yip1-6* and *yip1-4*, that render a physiological consequence to the cell would interact with Rab-GDI. For these experiments, I GFP-tagged the mutant versions of the soluble domain of Yip1p and cotransformed them into yeast with a vector containing GST-GDI. Pulldown experiments were then performed in the same fashion as described above. The results are presented in Figure 5. We found that *yip1-6* and *yip1-4* bind Rab GDI in lysates more strongly than the wild type counterpart. This suggests that the defect associated with these alleles lies in the association of Yip1p and Rab-GDI. Corroborating these data are the results in Figure 6. Yip1p associates with Yif1p another member of the YIP1 family (3,14,15). The overexpression of both of these proteins leads to a dominant negative phenotype (Barlowe laboratory, unpublished results). This is demonstrated in Figure 6. In yeast expressing Yip1p/Yif1p under the control of the Galactose promoter, the cells fail to grow in galactose but grow in glucose. However, centromeric or multicopy episomal plasmids containing yeast Rab-GDI (Gdi1p) are able to suppresses this phenotype suggesting that Rab-GDI compensates for the Yip1/Yif1 complex overexpression. This may mean that Rab GDI and Yip1p/Yif1p complex act antagonistically. Further experiments characterizing *yip1-6* and Rab-GDI are under way in our laboratory.

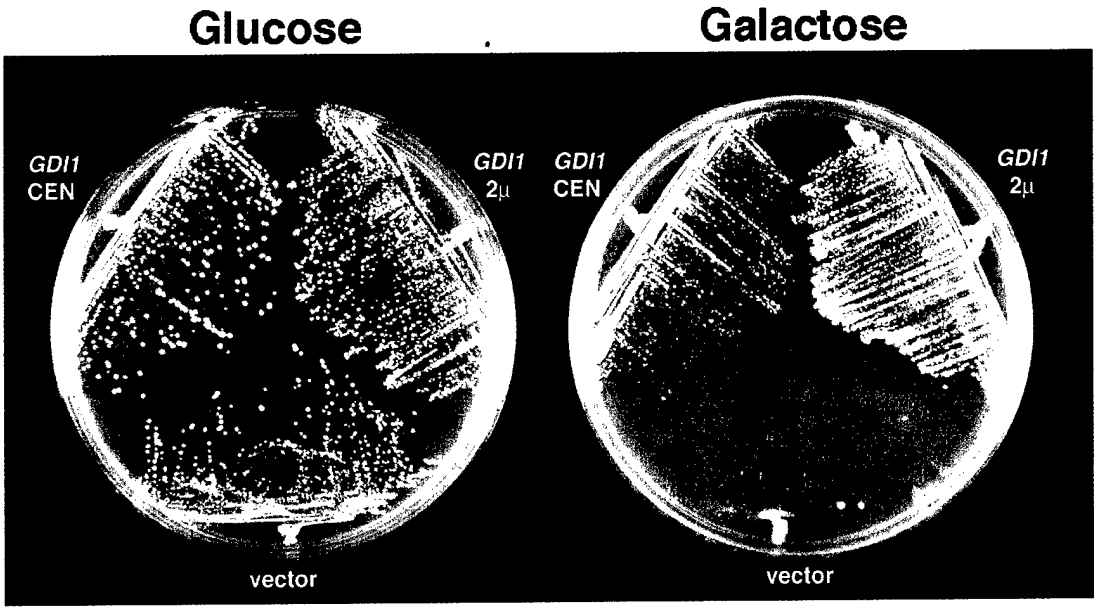


Figure 6 Yip/Yif complex overexpression is dominant negative and *GDI1* can suppress this phenotype. A strain containing integrated $P_{Gal1/10}$ *YIP1* and *YIF1* was kindly provided by Barlowe laboratory. This strain fails to grow in galactose containing media due to the overexpression of the Yip1p and Yif1p proteins. An episomal vector (either a CEN or 2μ) containing *GDI1* rescues this phenotype.

Yip1p affects the localization of GFP-Ypt1p while sec19-1 does not.

In the appended manuscript , I present data suggesting that Yip1p, alters the localization of one of the Rabs Ypt1p. Ypt1p resides primarily in the Golgi but loss of Yip1p's function leads to the cytoplamic distribution of Ypt1p. In contrast, loss of Gdi1p function in the original *sec19-1* strain, does not lead to the loss of the Golgi apparatus localization of GFP-Ypt1p (Figure 7). This suggests that possibly *sec19-1* defect lies on the inability of Gdi1p to extract Rabs off membranes. Data in our laboratory suggests that *yip1-4* and *sec19-1* are synthetically lethal (Catherine Chen, unpublished data). If Yip1p function lies on recruiting Ypt1p onto membranes, having a defect in loading (*yip1-4*) and extraction (*sec19-1*) off membranes would create a lethal situation at the permissive temperatures of both mutants.

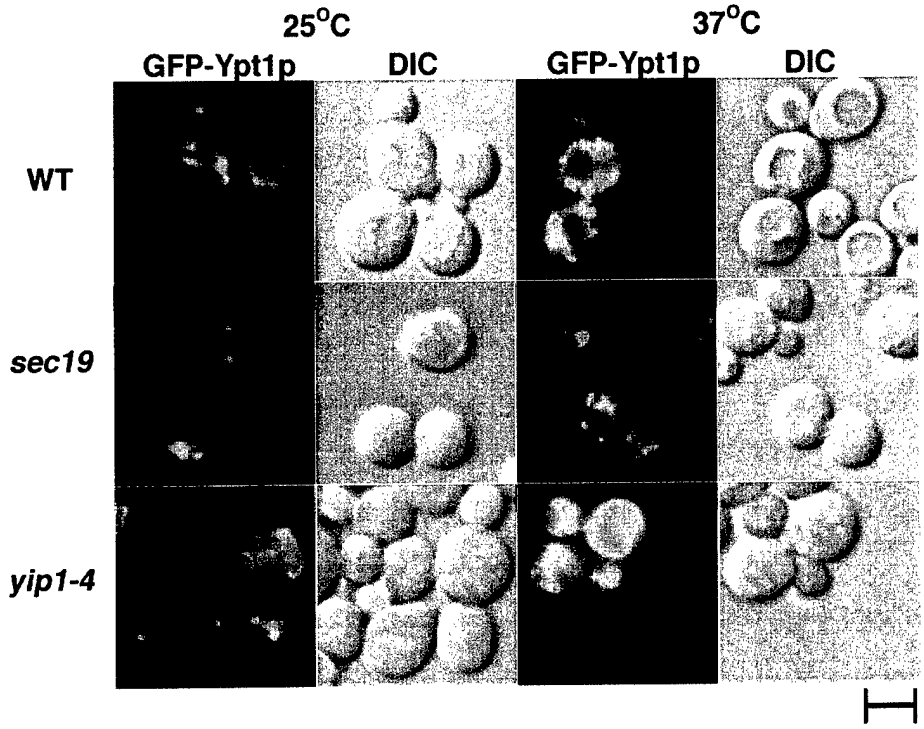


Figure 7 *yip1-4* mutant cells are defective in Golgi localization of the Rab protein Ypt1p at the restrictive temperature but *sec19-1* are not.

The localization of GFP-Ypt1p was measured in *yip1-4* and *sec19-1* mutant and wildtype cells following a shift to the restrictive temperature (37°C) for 30 minutes. The cells were visualized by fluorescence microscopy. The left side of each panel shows GFP fluorescence while the right side is DIC optics. The characteristic punctate Golgi distribution of GFP-Ypt1p becomes diffuse at 37°C and cytosolic in *yip1-4* mutant cells but not in *sec19-1* mutant cells or wild type cells.

c) Cloning of mammalian homologs: I have cloned the conditional mutants of the mammalian homologs of Yip1 into mammalian expression vectors. The idea is to characterize them in cancer cell lines. I transfected these constructs into mammalian cells and the results although preliminary appear to suggest the dominant negative Yip1p inhibits vesicular transport, a finding that was already published by Tang and colleagues. Further work would have to reveal the specific role of these intriguing proteins and their role in uncontrolled proliferation and cancer.

KEY RESEARCH ACCOMPLISHMENTS OF LAST YEAR:

- Discover that double prenylation is essential for Rab protein localization and function.
- Insights into Yip1p function: genetic, biochemical and cell biological data suggest that Yip1p recruits Rab GTPases onto membranes and therefore might play a crucial role in Rab protein activation.
- Clone conditional mutants of Yip1p and the mammalian homologs.

KEY RESEARCH ACCOMPLISHMENTS OF PREVIOUS YEARS:

- Investigation of the phenotypes and morphological alterations of cells overexpressing YOP1
- Physical characterization of Yop1p.
- Biochemical and genetic analysis of Yop1p/Yip1p interactions
- Analysis of Yop1/Rab interactions
- Localization of Yop1p in yeast.
- Cloning of Yop1p and TB2 into expression vectors to begin antibody preparation.
- Characterization of the Yip3p, yeast homolog of PRA1, a protein identified to associate with Rab3A
- Characterization and identification of Yip1p family members in yeast.
- Cloning of mammalian Yip1A (homolog of Yip1p). Two hybrid data indicating that mammalian Yip1A associates with mammalian Rabs.
- Co-localization data of Yip1A with yeast Yip1A in mammalian cells.
- Identification of the di-geranylgeranylation of Rab proteins as a requirement for localization and function.
- Yip1p specifically interacts with doubly prenylated Rabs.
- Cloning of mammalian Rab3A, Rab8, Rab13 and mammalian homolog of Yop1p (TB2).

REPORTABLE OUTCOMES:

Outcomes that have resulted from this research :

1. Journal article publication: Calero, M., Chen, C. Z., W., Z., Winand, N., Havas, K. A., Gilbert, P. M., Burd, C. G., and Collins, R. N. (2003) "Double prenylation is essential for Rab protein localization and function" ***MBC*** **14**(5):1852-67.

2. Journal article publication : Calero,M. and Collins,R.N..(2002) "Saccharomyces cerevisiae Pra1p/Yip3p Interacts with Yip1p and Rab Proteins". ***Biochem Biophys Res Commun*** **290**(2): 676-81.

3. Journal article publication : Calero,M., Winand, N.J. and Collins,R.N..(2002) "Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors". ***FEBS Lett.*** **515**(1-3):89-98.

4. Journal article in preparation: Calero,M., Whittaker,G.W and Collins,R.N..(2001) "Yop1p, the yeast homolog of the polyposis locus protein 1, interacts with Yip1p and negatively regulates cell growth" ***J Biol Chem*** **13;276**(15):12100-12.

5. Poster presented at the ERA OF HOPE DOD meeting in Orlando Florida 2002.

6. Abstract: Calero,M,Whittaker,G.W. and Collins,R.N "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p" *MCB* Abstracts for ASCB meeting. Poster presented at the 40th Annual Meeting of The American Society for Cell Biology (ASCB): "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p"

7. Poster presented at the annual meeting of the field of Pharmacology, Cornell University: "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p"

CONCLUSIONS:

The ultimate goal of my research is to elucidate the role of TB2 and its relationship with Rab GTPases. There is a growing appreciation that many proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation by diverse mechanisms (16-18). It is not known whether TB2 (Yop1p homolog) or Yip1p are directly linked to tumorigenesis, as the majority of studies have examined the roles of the two adjacent genes MCC and APC . Our genetic data in the past, suggested that *YOP1* has the properties expected of a tumor suppressor gene, which negatively regulates cell growth. Our data indicate that *YOP1* and possibly TB2 may play a role in the regulation of cell growth through its facilitation of membrane traffic. However, much needed to be understood about these proteins. During the progress of my research, I have defined the YIP1 family and discover insights into their possible function.

In this report, I have presented evidence suggesting that Yip1p may act in an antagonistic way to Rab-GDI. Firstly, Yip1p being a membrane bound protein, it displays very similar interactions with Rab GTPases as Rab-GDI. The only difference is that Yip1p requires the double prenylation for the association with Rabs whilst Rab-GDI can associate with singly prenylated Rabs such as Rab8 and Rab13 (appended manuscript). Secondly, the soluble domain of Yip1p is able to associate in cellular yeast lysates with Rab GDI. The biological importance of this interaction is underscored by the fact that two alleles of YIP1 (*yip1-4* and *yip1-6*) that have conditional lethal phenotypes associate stronger with Rab GDI. Thirdly, I present genetic evidence suggesting that Rab-GDI is able to suppress the dominant negative phenotype of the overexpression of the Yip1p/Yif1p complex. Fourthly, cell biological data presented in Figure 7 suggests that the membrane localization of GFP-Ypt1p is dependent upon Yip1p function while it remains unaffected by the loss of Rab-GDI function (as assessed in the *sec19-1* strain). These results together with the genetic evidence that *yip1-4* and *sec19-1* are synthetically lethal (unpublished results), suggest that Yip1p and Rab-GDI act in the same pathway and have antagonistic functions. Therefore, Yip1p and related family members may have a crucial role in the activation of Rab GTPases.

A major goal in the original statement of work was to elucidate the role of Yip1p and related proteins. Although, my work has suggested a possible involvement of Yip1 proteins in the regulation of Rab GTPases, much remains to be understood. An original impetus of my proposal was to establish a system in which I could characterize the role of these proteins in cancer cell lines. However, the reality was that not enough information was known and understood about Yip1 and yeast served as an excellent model to start the analysis of the function of these proteins. My work, has open the field of these new regulators of Rab function and further studies would need to address the potential role in cancer.

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Yop1p, the Yeast Homolog of the Polyposis Locus Protein 1, Interacts with Yip1p and Negatively Regulates Cell Growth*

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Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. We have recently characterized a protein, Yip1p, which appears to play a role in Rab-mediated membrane transport in *Saccharomyces cerevisiae*. In this study, we report the identification of a Yip1p-associated protein, Yop1p. Yop1p is a membrane protein with a hydrophilic region at its N terminus through which it interacts specifically with the cytosolic domain of Yip1p. Yop1p could also be coprecipitated with Rab proteins from total cellular lysates. The *TB2* gene is the human homolog of Yop1p (Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levey, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H. M., Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) *Science* 253, 661–665). Our data demonstrate that Yop1p negatively regulates cell growth. Disruption of *YOP1* has no apparent effect on cell viability, while overexpression results in cell death, accumulation of internal cell membranes, and a block in membrane traffic. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic.

The Rab family encompasses a conserved group of key molecules involved in membrane traffic and represents a distinct subgroup of the Ras superfamily (2). Each stage of membrane traffic through both the constitutive and regulated secretory pathways of all eukaryotic cells is associated with a distinct Rab protein that regulates the cascade of events that lead to SNARE-mediated membrane fusion (3). A hallmark of Rabs is their localization to specific compartments of the transport pathway. This distribution is consistent with the function of Rab proteins in distinct intracellular transport processes. In every case examined, the localization pattern of a Rab protein reflects the membrane transport step that it regulates. In keeping with this view, more than 30 members of the Rab family have been identified (2).

Rabs are stably prenylated at their C terminus, which mediates their association with membranes (4). However, while the majority of Rabs are membrane-associated, prenylated Rabs are also found in the cytosol bound to the Rab GDP dissociation inhibitor (GDI).[1] GDI shares sequence homology with the Rab escort protein involved in presenting and removing Rab proteins from the prenylation machinery (5, 6). GDI has several properties that underscore its role in mediating Rab protein function: (i) GDI binds preferentially to the GDP-bound conformation of Rab proteins and slows the intrinsic rate of GDP nucleotide dissociation (7), (ii) GDI requires the fully prenylated Rab protein for interaction and binds in such a way so that the geranylgeranyl groups are shielded in a hydrophobic pocket (8), (iii) GDI is a pleiotropic factor interacting with many different Rab proteins *in vitro* and *in vivo*; in *Saccharomyces cerevisiae*, a single gene encodes GDI function for all 11 Rab proteins (9). These properties enable the Rab protein to exist in the aqueous environment of the cytoplasm as a soluble heterodimer with GDI and facilitate recycling of the GDP-bound Rab back to the donor compartment (10). Consistent with this model, Rab proteins are complexed to GDI in the cytosol, and depletion of GDI in yeast causes loss of the soluble pool of Rabs and a concomitant inhibition of transport in the secretory pathway.

The specificity of Rab protein function, localization, and their presence on the surface of vesicles suggests the existence of a machinery that recruits Rab proteins to the proper target membrane. However, identification of such a machinery has proven elusive. To date, no factor mediating this process has been identified; however, several features of Rab membrane recruitment have been established: (i) Rabs are recruited to membranes in their inactive GDP-bound conformation bound to GDI (11); (ii) membrane recruitment is accompanied by the displacement of GDI (12); (iii) membrane recruitment is specific, and the C-terminal hypervariable region of the Rab protein mediates this specificity (13); (iv) prenylation of Rab proteins is crucial for membrane recruitment in addition to the C-terminal ~35 amino acid residues; (v) membrane recruitment is followed by nucleotide exchange, and the two processes can be distinguished kinetically (14, 15); and (vi) for Rab4, the existence of a membrane protein that acts as a specific Rab receptor has been demonstrated, although the precise identity of this receptor is unknown (16).

We have characterized a membrane protein in yeast, Yip1p, which appears to mediate the dissociation of the Rab het-

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[1] The abbreviations used are: GDI, GDP dissociation inhibitor; GST, glutathione *S*-transferase; HA, influenza virus hemagglutinin epitope; PCR, polymerase chain reaction; ORF, open reading frame; PBS, phosphate-buffered saline; PNS, postnuclear supernatant; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CPY, carboxypeptidase Y.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
NY605	*MAT***a** *ura3–52 leu2–3,112*	Novick laboratory
RCY376	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*HA-YOP1*	This study
RCY377	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*HA-YOP1* C terminus	This study
RCY404	*MAT***a** *leu2–3,112 ura3–52 YOP1*::*URA3 GFP-YOP1*	This study
RCY407	*MAT***a** *ura3–52 leu2–3,112 YOP1ΔKAN*^{R}	This study
RCY423	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YOP*	This study
RCY425	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YOP1* [URA3 2μ myc$_9$-*YIP1*]	This study
RCY427	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST*	This study
RCY428	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST* [*URA3* 2μ myc$_9$-*YIP1*]	This study
RCY429	*MAT***a** *ura3–52 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YOP1* [*URA3* 2μ *DSS4*-myc$_3$]	This study
RCY460	*MAT***a** *ura3–52 leu2–3,112 YOP1ΔKAN*^{R} [*URA3 CEN HA-YOP1*]	This study
RCY462	*MAT***a** *ura3–52 leu2–3,112* [*URA3* 2μ myc$_9$-*YIP1*]	This study
RCY469	*MAT***a** *leu2–3,112 ura3–52*::*URA3 HA-YOP1 YOP1ΔKAN*^{R}	This study
RCY496	*MAT***a** *leu2–3,112 ura3–52*::*URA3 HA-YOP1* [*LEU2* CEN *GFP-YIP1*] *YOP1ΔKAN*^{R}	This study
RCY509	*MAT***a** *leu2–3,112 ura3–52* [*URA3* 2μ *GAL*$_{1-10}$→*GST-YOP1*] [*LEU2* CEN *GFP-YIP1*]	This study
RCY508	*MAT***a** *leu2–3,112 ura3–52* [*URA3* 2μ *GAL*$_{1-10}$→*GST-YOP1*] [*LEU2* CEN *GFP-YPT7*]	This study
RCY455	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YPT52* [pRS426 *GAL*$_{1-10}$→*HA-YOP1 (pRC782)*]	This study
RCY456	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-SEC4* [pRS426 *GAL*$_{1-10}$→*HA-YOP1 (pRC782)*]	This study
RCY457	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YPT1ΔC* [pRS426 *GAL*$_{1-10}$→*HA-YOP1 (pRC782)*]	This study
RCY465	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YPT6* [pRS316 *GAL*$_{1-10}$→*HA-YOP1 (pRC783)*]	This study
RCY467	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST-YPT7* [pRS316 *GAL*$_{1-10}$→*HA-YOP1 (pRC783)*]	This study
RCY464	*MAT***a**/α *ura3–52 his3Δ200 leu2–3,112*::*LEU2 GAL*$_{1-10}$→*GST* [pRS316 *GAL*$_{1-10}$→*HA-YOP1 (pRC783)*]	This study
Y190	*MAT***a** *gal4Δgal80Δ trp1–901 ade2–101 ura3–52 leu2–3,112 URA3*::*GAL10*→*LacZ, LYS2*::*GAL10*→*HIS3 cyh*^{R}	Elledge laboratory

erodimer from GDI. *YIP1* is an essential gene (17) that is highly conserved in evolution.[2] However, Yip1p is a pleiotropic factor and lacks specificity for interaction with any particular Rab GTPase (17). We have therefore searched for a protein accessory factor that may act in conjunction with Yip1p, and we report the identification of a novel membrane protein, Yop1p, which physically interacts with Yip1p. Disruption of *YOP1* has no apparent effect on cell viability, while overexpression results in cell death and accumulation of internal cell membranes. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic. Because of the essential nature of Rab recruitment for the activation and recycling of Rabs, characterization of Yop1p may provide crucial insight into the action of Rab proteins in mediating membrane transport.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The *S. cerevisiae* strains used in these studies are listed in Table I. All yeast strains were manipulated as described by Guthrie and Fink (18). *YOP1* gene deletion was carried out using the *KAN*^{R} module (19) as a selectable marker and the primers CAAAGACATAACCGCACTCCAATCATGTCCGAATATGCATCTAGTATTCACTCTCCGTACGCTGCAGGTCGAC and GAGGATATAGGTGAGTTGCCTCTTAATGAACAGAAGCACCTGTAGCCTTAGAAGCCTATCGATGAATTCGAGCTCG to precisely eliminate the *YOP1* ORF. Genomic PCR using an internal deletion primer and the flanking primer CTTGAAGCTTGTTATTCCGA was performed to verify gene disruption. Yeast expressing GST-Yop1p under the control of the $GAL_{1/10}$ promoter (RCY423) and GST alone (RCY427) were created by digesting pRC494 and pRC337, respectively, with *Cla*I to direct integration at the *LEU2* locus of NY605. Strains RCY425, RCY428, and RCY462 were created by transforming pRC695 into RCY423, RCY427, and NY605, respectively. In the same manner, RCY429 was created by transforming pNB632 into RCY423. RCY460 was created by transforming the hemagglutinin (HA)-tagged Yop1p protein expression vector pRC778 into RCY407. For immunofluorescence, RCY469 was created by digesting pRC833 with *Eco*RV to direct integration of the plasmid at the *URA3* locus of RCY407.

Yeast strains were streaked out on a selective plate and incubated at 30 °C. Liquid media cultures were grown at room temperature. A single colony from each strain was inoculated into 5 ml of selective medium and grown to stationary phase. The day prior to the experiment, medium was inoculated with aliquots of stationary culture at room temperature to obtain cells in logarithmic phase growth. Turbidity measurements were made using a Beckman model DU-40 spectrophotometer at 600 nm.

Plasmids and DNA Constructs—The genomic *YOP1* ORF contains a single intron. For convenience, this intron was removed for the majority of *YOP1* constructs by overlap PCR with the primers RNC66 (GGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGGAAGAGTTGCATAGATAGGATGGGTGA) and RNC78 (CGATACCAAGTACTCTGGTAATAGAATTTTACAGC) together with RNC67 (CTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATGCTCAAAAGCTAACACTAGGCCAG) and RNC79 (TATCCATGGGTAAGTACTCTGGTAATAGAATTTTACAGC). Full-length *YOP1* fusion constructs were constructed by PCR with oligonucleotides RNC44 (TGGTACCTCATGAGCGAATATGCATCTAGTATTCACTCTC) and RNC80 (AATAGGATCCTTAATGAACAGAAGCACCTGTAG). The *Nco*I/*Bam*HI-digested PCR product was subcloned into pAS2-1 and pACT2 to create two-hybrid vectors expressing full-length *YOP1*, p121 to create HA-tagged *YOP1* under the control of the $GAL_{1/10}$ promoter (pRC393) and pRC337 to create GST-tagged *YOP1* under the control of the $GAL_{1/10}$ promoter (pRC494). C-terminal *YOP1* constructs containing amino acids 18–180 were created in a similar manner with the oligonucleotides RNC79 (TATCCATGGGTAAGTACTCTGGTAATAGAATTTTACAGC) and RNC80. The PCR product was subcloned into p121 to create pRC439 expressing HA-tagged *YOP1* C terminus under the control of the $GAL_{1/10}$ promoter. pRC581 containing yEGFP-tagged *YOP1* under the control of its own gene regulatory elements in pRS406 was created by overlap PCR with the oligonucleotides RNC66, RNC67, RNC179 (CAAAGACATAACCGCACTCCAATCATGTCTAAAGGTGAAGAATTATTC), RNC180 (AGAGTGAATACTAGATGCATATTCGGATTTGTACAATTCATCCATACC), RNC181 (CATGATTGGAGTGCGGTTATG), and RNC182 (TCCGAATATGCATCTAGTATTCACTCTCAAATGAAAC). pRC695 expressing Myc$_9$-*YIP1* in pRS426 (20) was created by overlap PCR placing a cassette containing Myc$_9$ (gift of Y. Barral, ETH, Zurich) in frame behind the start codon of *YIP1* with the primers 9× oligo 1 YIP1 (GCAAGACAACTATTAGTCCCTCTCGAGATGCTCCACCGCGGTGGC) and 9× oligo 2 YIP1 (TGTTACTAGTATTGTAGAAAGACATAATTCCTGCAGCCCGGGGGAT). pNB632, a *URA3* multicopy plasmid containing *DSS4*-Myc$_3$, has been described previously (21). pRC337 was created by subcloning GST in front of the $GAL_{1/10}$ promoter of vector pNB527 digested with *Bam*HI/*Xho*I using primers RNC177 (CTAGACTAGAT-

[2] R. Collins, unpublished data.

CTTCATGAGTTCCCCTATACTAGGTTATTGGAAAATTAAG) and RNC178 (GACTGACCTCGAGTAGGATCCAGTCACCATGGTCAGATCCGATTTTGGAGGATG) and digesting the PCR product with *Bgl*II/*Xho*I. pRC778 containing a single HA epitope at the N terminus of Yop1p expressed at wild-type levels in the vector pRS315 was created by PCR overlap with the oligonucleotides RNC157 (TACGACGTCCCAGACTACGCTTCCGAATATGCATCTAGTATTCAC) and HA 1 (AGCGTAGTCTGGGACGTCGTATGGGTACATCTCGAGAGGGACTAATAGTTGTC). The insert of pRC778 was removed with *Sal*I/*Hin*dIII and ligated into the vector pRS306 digested with *Xho*I/*Hin*dIII to create pRC833, a *URA3*-integrating vector expressing wild-type levels of HA-tagged Yop1p. pRC693 containing green fluorescent protein (GFP)-tagged Yip1p under the control of its own promoter and terminator in pRS315 was constructed by placing a cassette containing yeast-enhanced GFP mut3 (22) in frame behind the start codon of *YIP1* with the primers GFP oligo 1 (GACAACTATTAGTCCCTCTCGAGATGTCTAAAGGTGAAGAATTATTCAC) and GFP oligo 2 (GTTACTAGTATTGTAGAAAGACATTTTGTACAATTCATCCATACCAT). pRC650 and pRC556 containing GFP-tagged Ypt6p and Sec4p, respectively, in pRS315 were created in a similar fashion. pRC903 was created by subcloning a cassette containing GST-tagged *YOP1* under the control of the $GAL_{1/10}$ promoter from pRC494 into the 2μ *URA3* vector pRS426. pRC940 containing *YIP1* was constructed by genomic PCR with the oligonucleotides YF YIP1 (GTACCGGGCCCCCCCTCGAGGTCGACGTAGTGCTTGTTACGTTAG) and YR YIP1 (CCACCGCGGTGGCGGCCGCTCTAGAACTCTATGCTTTCCTTATTTACCTCTGGA) and inserted into pRS426 to create a multicopy *URA3* vector.

Electrophoresis and Western Blotting—For electrophoresis, samples were boiled for 5 min in gel loading buffer (60 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromphenol blue), microcentrifuged for 5 min, and loaded onto 12 or 14% SDS-polyacrylamide gels (37.5:1 acrylamide/bisacrylamide). Prestained protein molecular weight markers were from Life Technologies, Inc. For Western blotting, gels were transferred to polyvinylidene difluoride membranes for 2 h at 200 mA. The membranes were stained with Ponceau S to observe the quality of the transfer. Antigens on the membrane were detected by incubating the filter with blocking buffer (5% nonfat dry milk in TBST; 150 mM NaCl, 50 mM Tris, pH 7.5, and 0.2% Tween 20). Primary antibodies were incubated in TBST, followed by three washes. Secondary alkaline phosphatase-conjugated antibodies were added in blocking buffer, followed by three washes and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

Coprecipitation Assays—Yeast strains were grown in minimal medium containing 2% galactose. 10 OD units from each culture were harvested and washed in 1 ml of ice-cold TAZ buffer (10 mM Tris, pH 7.5, 10 mM NaN_3). Cell pellets were then resuspended in 100 μl of ice-cold lysis buffer (20 mM KPi, 80 mM KCl, 1 mM EDTA, 2% glycerol, 0.1% Tween 20) containing protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A), and an equal volume of glass beads was added. The cells were then lysed by vortexing for 2 min in a Turbo-Beater (Fisher) at 4 °C. A total detergent-solubilized lysate was generated by incubating lysates end-over-end with an additional 1 ml of lysis buffer for 10 min at 4 °C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microcentrifuge for 5 min at 13,000 rpm. 20 μl of glutathione *S*-transferase 4B beads (GST-beads; Amersham Pharmacia Biotech) was added to the lysates and incubated with constant mixing for 30 min at 4 °C. After four washes with 0.6 ml of lysis buffer, the GST beads were boiled with SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE and Western blot. Pull-down experiments from yeast strains RCY509 and RCY508 used the lysis buffer 25 mM KPi, pH 7.5, 160 mM KCl, 2 mM EDTA, 2% glycerol, and 0.4% Triton X-100. Pull-down experiments fom yeast strains RCY455, RCY456, RCY457, RCY465, RCY467, and RCY464 used the lysis buffer, 25 mM KPi, pH 7.5, 160 mM KCl, 2 mM EGTA, 2% glycerol, and 0.5% Tween 20. Primary antibodies used were rabbit polyclonal α-GST (gift of T. Fox, Cornell University), mouse monoclonal α-Myc antibody (9E10; Ref. 23), affinity-purified Rabbit α-GFP antibody (24) (gift of P. Silver, Dana-Farber Cancer Institute), and mouse monoclonal α-HA 12CA5. Alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies were used (Bio-Rad) to detect the presence of Myc_9-Yip1p and either GST alone or GST-Yop1p.

Subcellular Fractionation—Yeast strain RCY460 containing wild-type levels of HA-tagged Yop1p as the only source of *YOP1* was used for this experiment. 25 OD units were harvested and washed in 1 ml of TAZ buffer. Cells were broken by glass bead lysis in a Turbo-Beater at 4 °C in fractionation buffer with protease inhibitors (PBS containing 0.2 M sorbitol and 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin). A postnuclear supernatant (PNS) was generated by two sequential centrifugation steps for 5 min at 500 × *g*. 2.7 mg of PNS was then spun sequentially at 10,000 × *g* for 15 min and at 100,000 × *g* for 12 min to generate P10 and P100 fractions. For Triton X-100 solubilization, the P100 membrane pellet was resuspended in fractionation buffer containing 1% Triton X-100. Samples were incubated for 10 min on ice and recentrifuged at 100,000 × *g*. For high salt treatment, the P100 membrane pellet was resuspended in fractionation buffer containing 1 M NaCl. Samples were incubated for 10 min on ice and recentrifuged at 100,000 × *g*. Pellets and supernatants were resuspended in sample buffer and analyzed by SDS-PAGE and Western blot. The HA-Yop1p was detected with mouse monoclonal 12CA5 antibody followed by anti-mouse alkaline phosphatase-conjugated secondary antibody.

Triton X-114 Phase Separation—Triton X-114 (Roche Molecular Biochemicals) was purified by precondensation as described (25). 25 OD units of yeast strain RCY460 were harvested and washed in 1 ml of TAZ buffer. Postnuclear supernatants were generated as described above. 1.8 mg of PNS was added to the same volume of PBS containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A). The samples were incubated for 20 min at 4 °C to solubilize membrane proteins. The lysates were incubated for 3 min at 30 °C followed by low speed centrifugation (700 × *g*) to separate the detergent-enriched and the soluble phases. This cycle was repeated a further two times with the detergent-enriched and soluble phases individually. The detergent phase was washed twice with PBS containing 0.05% Triton X-114 and the soluble phase with 10% Triton X-114. Samples were analyzed by SDS-PAGE and Western blot. Snc1/2p, an integral membrane protein, was used as a positive control and was detected with anti-Snc1/2p antisera (gift of P. Brennwald, Cornell University).

Carboxypeptidase Y Analysis—Yeast strains RCY376 and RCY377 containing HA-YOP1 full-length and C-terminal constructs (respectively) behind the galactose promoter, were grown in sucrose minimal medium to early log phase, before washing and resuspending in galactose minimal medium. At the indicated intervals, aliquots of 5 OD units were harvested for production of lysates. For the *sec18* experiments, cells were grown at room temperature until log phase before shifting an aliquot to the restrictive temperature (37 °C) for 1 h. Lysates were then boiled with SDS-PAGE sample buffer for 5 min and analyzed by SDS-PAGE and Western blot. The membrane was probed with polyclonal anti-carboxypeptidase Y (CPY) (gift from P. Brennwald).

Immunofluorescence Experiments—Yeast strains RCY469 containing HA-Yop1p and RCY407 (isogenic untagged control) were grown to early log phase in YPD medium. 2× fixative (2× PBS, 4% glucose, 40 mM EGTA, 7.4% formaldehyde) was added to an equal volume of medium containing 3 OD units of cells and incubated for 20 min at room temperature. Cells were then collected by centrifugation, resuspended in 5 ml of 1× fixative, and incubated for a further 1 h. The cells were washed twice in 2 ml of spheroplasting buffer (100 mM KP_i, pH 7.5, 1.2 M sorbitol) and then incubated in spheroplasting buffer containing 0.2% 2-mercaptoethanol and 0.08 mg/ml of zymolyase for 30 min at 37 °C with gentle mixing. 20 μl of the cell suspension was placed on individual wells of a polylysine-coated printed microscope slides (Carlson Scientific, Inc.) for 10 min. The cells were then washed three times with

TABLE II
Pattern of two-hybrid interactions of YOP1 with various YIP1 constructs

β-Galactosidase activity was determined by filter assay. Pairs were coexpressed in the reporter strain Y190. Plus represents a positive activity rated according to the following criteria: +++, activity detected after 30 min; ++, activity detected after 90 min; and +, activity detected after 5 h. −, a negative indication of activity. At least 30 independent transformants were tested for each pair. Yop1p N-terminal constructs contain amino acids 1–17, and Yop1p C-terminal constructs contain amino acids 18–180. Yip1p N-terminal constructs contain amino acids 1–117. ND, not detected.

Bait construct	Fish construct			
	Yip1p N-terminus	Yip1p full length	Yop1p N-terminus	Yop1p full length
Yip1p N terminus	−	−	+++	ND
Yip1p full length	−	−	+++	−
Yop1p N terminus	+++	+++	−	−
Yop1p C terminus	−	−	−	−

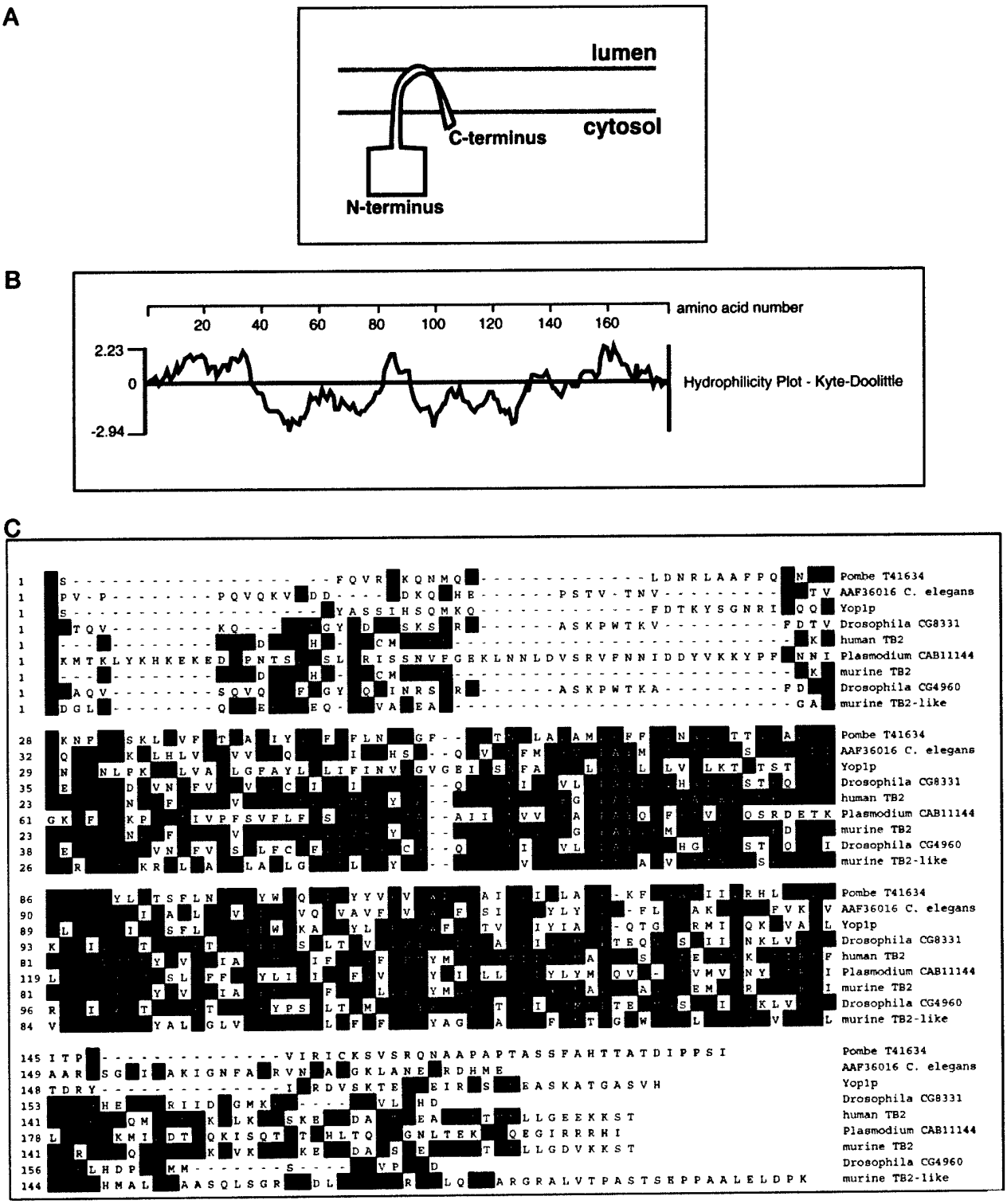


FIG. 1. *A*, schematic representation of Yop1p. Sequence data indicate a cytoplasmically oriented N terminus and a hydrophobic C-terminal domain that spans the membrane twice. B, Kyte-Doolittle hydrophobicity plot of Yop1p. This was generated using the program Protean (DNASTAR) with a 9-residue parameter average and shows the relative location of the two hydrophobic segments of the protein. *C*, alignment of Yop1p with data base homologs. Shown is the sequence of Yop1p and a comparison with human and murine TB2 and full-length cDNAs from other organisms. T41634 is from *Schizosaccharomyces pombe*, CG4960 and CG8331 are from *Drosophila melanogaster*, AAF36016 is from *Caenorhabditis elegans*, and CAN11144 is from *Plasmodium falciparum*. Mammalian expressed sequence tag fragments are not included in this alignment. The sequences were aligned using MegAlign. Amino acid residues are numbered according to the protein sequence. The *shaded residues* exactly match the consensus sequence.

PBS/BSA (1 mg/ml BSA) and permeabilized for 5 min with either 0.1% SDS or 0.1% Triton X-100 in PBS/BSA. After washing five times in PBS/BSA, cells were blocked for 30 min in PBS/BSA. Polyclonal α-HA antibody (Y11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each well at a dilution of 1:5000 and incubated for 1 h at room temperature. Cells were washed 5 times in PBS/BSA and then incubated with Texas Red-labeled anti-rabbit secondary antibody (Molecular Probes, Inc.) at a dilution of 1:200 for 30 min at room temperature. Monoclonal 1.2.3 antibody was used to detect Sec4p (26), and a monoclonal anti-GFP antibody (3E6; Molecular Probes, Inc., Eugene, OR)

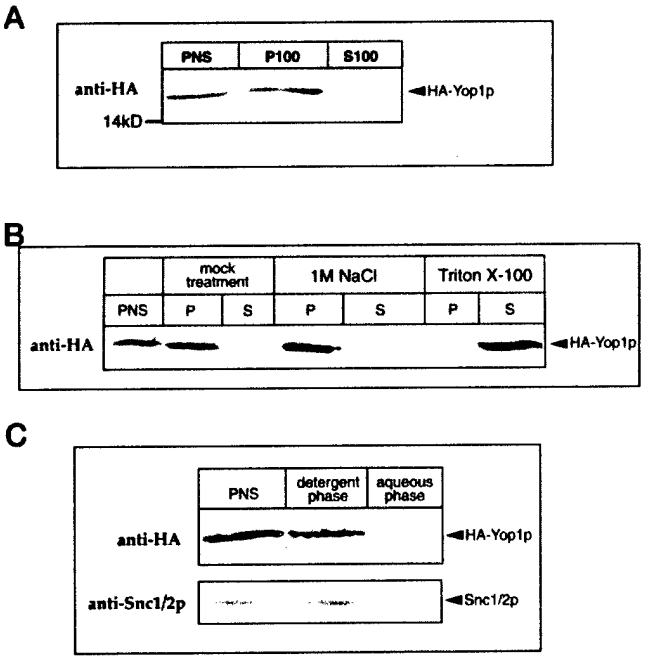


FIG. 2. *A*, membrane localization of Yop1p. Differential centrifugation was performed on logarithmically growing cells expressing HA-Yop1p at wild-type levels. Blots were probed for HA-Yop1p, which is present exclusively in the pellet fraction (P100; 100,000 × *g*). PNS represents a total postnuclear lysate (500 × *g* supernatant), and S100 is the supernatant remaining after the 100,000 × *g* centrifugation. *B*, high salt and Triton X-100 treatment of Yop1p-containing membranes. Postnuclear supernatants were centrifuged at 100,000 × *g* to obtain cytosolic and total membrane fractions. Total membrane fractions were resuspended in buffer with and without 1 M NaCl, 1% Triton X-100, or mock-treated before recentrifugation at 100,000 × *g*. The pellets and supernatants were dissolved in equivalent volumes of sample buffer and run on SDS-PAGE gel, and HA-Yop1p was detected by Western blotting. Relevant protein marker sizes are indicated. *C*, Triton X-114 phase separation of lysates expressing HA-tagged Yop1p. Triton X-114 fractionation generating a detergent-enriched phase and an aqueous phase was performed as described under "Experimental Procedures" on cells expressing HA-Yop1p at wild type levels. HA-Yop1p was detected by Western blotting and fractionates in the detergent-enriched phase. PNS represents total postnuclear supernatant. As a control, the fractions were probed for the membrane protein Snc1/2p, which is contained in the detergent-enriched phase. Relevant protein marker sizes are indicated on the *left*.

was used to detect GFP. These were followed by Oregon Green 514-labeled anti-mouse secondary antibody (Molecular Probes) at a dilution of 1:250. To stain nuclei, 5 μg/ml Hoechst 33258 (Molecular Probes) in PBS/BSA was added to each well, and after 10 min at room temperature, cells were washed five times. Cells were mounted in a small drop of mounting medium (Moviol), and the slides were left to air dry in the dark for at least 30 min. Confocal microscopy was performed using an Olympus FluoView confocal station (Olympus). Oregon Green was excited with the 488-nm line of an argon laser, and Texas Red was excited with the 568-nm line of a krypton laser.

Electron Microscopy—The cells were incubated for 14 h in medium containing galactose as sole carbon source at a final cell density (A_{600}) of between 0.4 and 0.7. Cells were washed with 0.1 M cacodylate, pH 6.8, and then fixed with 0.1 M cacodylate, pH 6.8, containing 3% glutaraldehyde for 1 h at room temperature and then overnight at 4 °C. The cell walls were removed by treatment with 0.1 M KP_i buffer, pH 7.5, containing 0.2 mg/ml zymolyase 100T. The cell pellet was incubated with 1.5 ml of cold 2% OsO_4 in 0.1 M cacodylate buffer for 1 h on ice followed by incubation with 1.5 ml of filtered 2% uranyl acetate (aqueous) at room temperature for 1 h. The cell pellets were dehydrated with the following ethanol washes: 50, 70, 90, and 100% followed by four washes from a fresh bottle of 200 proof ethanol and a final rinse in 100% acetone. The pellet was then incubated with 50% acetone, 50% SPURR resin (Electron Microscopy Sciences); this was changed to 100% SPURR resin, and the sample was transferred to beem capsules (Electron Microscopy Sciences) and baked at 80 °C for at least 24 h. Thin sections were cut onto Specimen Grids (Veco) (3-mm diameter, 75 × 300 mesh copper), contrasted with lead citrate and uranyl acetate, and then examined in an FEI Philips TECHNAI 12 BioTwin electron microscope at 100 or 80 kV.

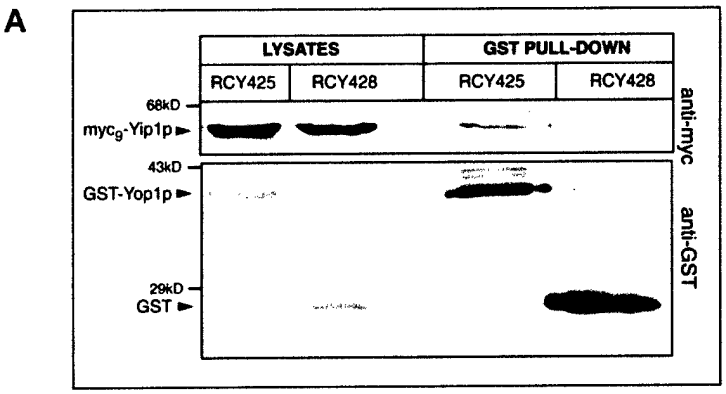
Two-hybrid Experiments—The ORF sequences were subcloned into pAS1-CYH2 or pAS2–1 for "bait" and pACTII for "fish" constructs, respectively. The yeast strain Y190 was used for to screen the library for N-terminal Yip1p-interacting clones (27). The yeast reporter strain Y190, which contains the reporter genes *lacZ* and *HIS3* downstream of the binding sequences for Gal4, was sequentially transformed with the pACT2 and pAS2–1 (CLONTECH) plasmids containing the genes of interest. Double transformants were plated on selective medium (lacking tryptophan and leucine) and incubated for 2–3 days at 30 °C. Trp+ Leu+ colonies processed for the β-galactosidase filter assay as described (21).

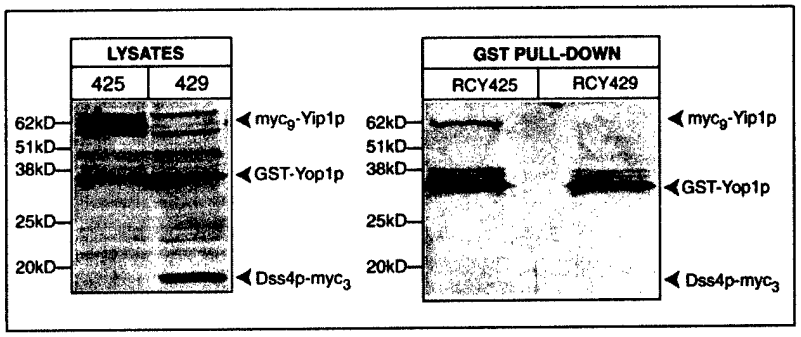
RESULTS

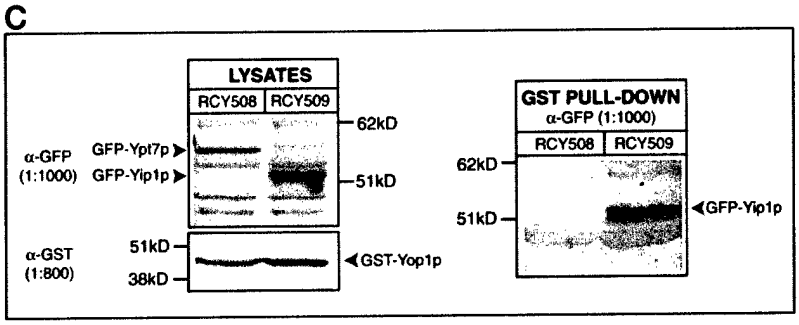
The Cytosolic Domain of Yip1p Interacts with a Novel Membrane Protein—To explore the role of Yip1p in membrane traffic, we considered the possibility that it may exist in physical association with other proteins. Such a protein may perhaps act to provide a specificity component to the Rab membrane recruitment reaction. To identify such potential proteins, we performed a two-hybrid screen using the cytosolic domain of Yip1p as bait. For this interaction screen, we used two-hybrid libraries constructed from short fragments (0.5–1 kilobase pair) of yeast genomic DNA (28). Since the yeast genome is relatively compact with few intron-containing genes, such a library represents a collection of random protein fragments. The rationale for such a strategy was that a Yip1p-interacting protein may be a membrane protein interacting with Yip1p through exposed soluble loops. Interactions may not be revealed by expressing full-length cDNAs, but protein fragments of the isolated loops alone may demonstrate interaction in the two-hybrid system. Analogous strategies have been used successfully to explore interactions of multispanning membrane proteins using the two-hybrid system (29). Using this screen, we identified a previously uncharacterized membrane protein derived from ORF *YPR028W*. The interacting clone identified contained 17 amino acids derived from the extreme N terminus of the protein fused in frame with the GAL4 DNA activation domain. We have termed this gene *YOP1* (YIP one partner). The interaction between the Yop1p fragment and Yip1p was recapitulated with a full-length Yip1p construct in the two-hybrid system. The interaction was also maintained whether or not the Yop1p fragment was a GAL4 DNA binding domain plasmid or a GAL4 DNA activation domain fusion; *i.e.* if the "bait" construct is swapped with the "fish" construct, the vast majority of false two-hybrid positives will not interact in such a test. However, Yop1p full-length constructs show no interactions with Yip1p in the two-hybrid system. These data are summarized in Table II.

The primary sequence of Yop1p is predicted to have at least two membrane-spanning domains (Fig. 1, *A* and *B*). A BLAST search of GenBank™ revealed that Yop1p is homologous to the human TB2 protein in addition to several other proteins present in data bases (Fig. 1*C*). Yop1p and human TB2 share 25.65% identity at the amino acid level, and there is 22.4% identity between Yop1p and murine TB2. It is notable that the overall structure of the mammalian and yeast protein is conserved. Both proteins contain extensive hydrophobic domains with the N terminus predicted to be exposed to the cytoplasmic face of the membrane. No other Yop1p homologs could be identified in *S. cerevisiae*.

Yop1p Is an Integral Membrane Protein—Sequence information predicts Yop1p to be a 20-kDa protein with two membrane-spanning segments that is likely oriented with its N terminus toward the cytoplasm (Fig. 1). We examined whether Yop1p has the expected properties of an integral membrane protein. First, Yop1p fractionated exclusively in the pellet of a total







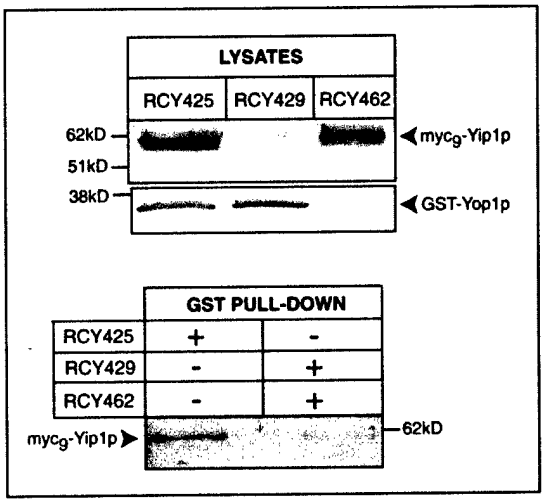


FIG. 3. *A*, biochemical analysis of Yop1p and Yip1p interaction: GST-Yop1p interacts specifically with Myc_9-Yip1p. Lysates were prepared from cells expressing either GST-Yop1p and Myc_9-Yip1p (RCY425) or GST and Myc_9-Yip1p (RCY428). Detergent-solubilized lysates were incubated with GST beads for 30 min at 4 °C as described under "Experimental Procedures." After four washes, the bead-bound material was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with both monoclonal 9E10 (1:500) to detect Myc_9-Yip1p and polyclonal anti-GST (1:800) to detect GST-Yop1p. Relevant protein marker sizes are indicated. Myc_9-Yip1p was detected on RCY425 but not on RCY428 after GST pull-downs. *B*, GST-Yop1p and Myc9-Yip1p interaction is specific to Yop1p and Yip1p. GST pull-down experiments as in *A* were performed on yeast expressing either GST-Yop1p and Myc_9-Yip1p (RCY425) or GST-Yop1p and Dss4p-Myc_3 (RCY429). The membrane was first probed with α-Myc to detect expression of Myc-tagged proteins and was subsequently probed with α-GST to confirm the presence of GST-Yop1p. Myc_9-Yip1p was detected after GST pull-down, but Dss4p-Myc_3 was not, indicating that the interaction is specific to Yip1p and Yop1p. *C*, GST-Yop1p specifically associates with Yip1p expressed at wild-type levels. Lysates were prepared from yeast expressing GST-Yop1p and either GFP-Yip1p (RCY509) or GFP-Ypt7p (RCY508) at single copy. Western blot analysis with affinity-purified anti-GFP antibody showed that GFP-Yip1p specifically associated with GST-Yop1p; however, a control protein (GFP-Ypt7p) expressed at similar levels did not associate with GST-Yop1p. Western blot of lysates demonstrates that the fusion proteins were expressed at equivalent levels in both strains. *D*, the complex of Yop1p and Yip1p interaction is formed *in vivo*. GST-Yop1p and Myc_9-Yip1p were either coexpressed in the same cell (RCY425) or expressed in different strains that were mixed after lysis (RCY429, RCY462). The GST-Yop1p and Myc_9-Yip1p interaction is only observed when the two constructs are expressed in the same cell, indicating that the interaction occurs prelysis or *in vivo*.

postnuclear supernatant centrifuged at 100,000 × *g*, indicating that it is either membrane-associated or present in a large pelletable aggregate (Fig. 2*A*). Second, we tested whether Yop1p was a peripheral membrane protein and could be removed by washing membranes in high salt-containing buffers. Yop1p could not be extracted from membranes by incubation in buffer containing 1 M NaCl; however, Yop1p was quantifiably extracted in Triton X-100 detergent-containing buffers

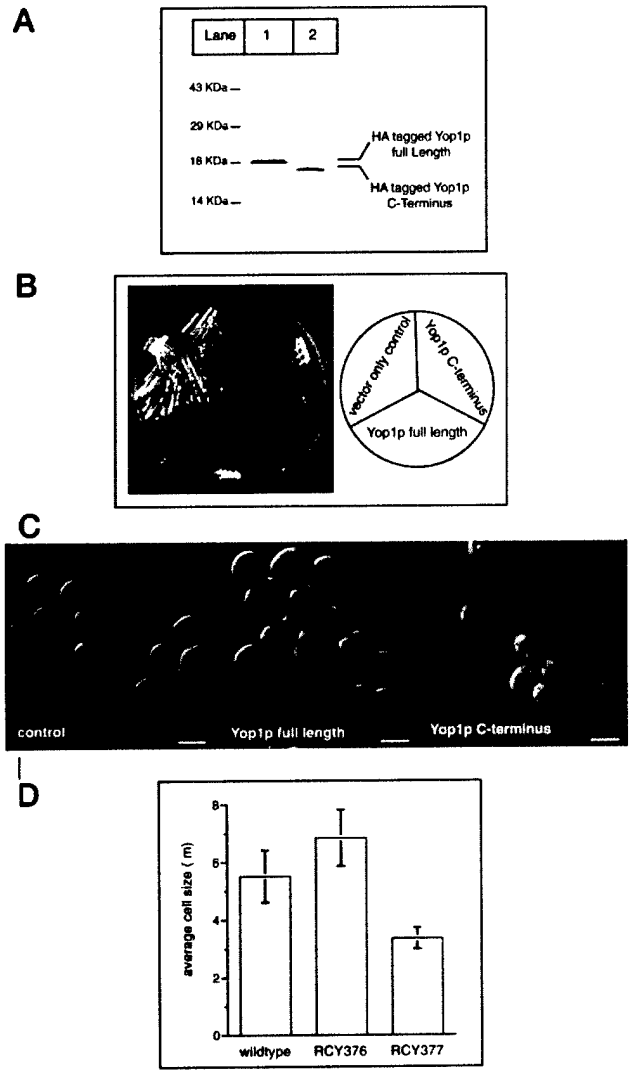


FIG. 4. **Overexpression of both Yop1p full-length and Yop1p C-terminal constructs results in a dominant negative phenotype.** *A* shows the ability of cells bearing constructs as indicated for galactose-dependent expression to grow when expression of the construct is induced by growth on galactose-containing medium after 3.5 days of growth at 30 °C. *B* shows a Western blot of lysates derived from cells shifted to galactose-containing medium for 10 h probed for the presence of the HA-tagged construct. *Lane 1* shows a lysate generated from RCY376 (Yop1p full-length construct), and *lane 2* shows a lysate generated from RCY377 (Yop1p C-terminal construct). *C* shows differential interference contrast (*DIC*) images of cells expressing the constructs as indicated and allowed to grow in galactose-containing medium for 2 days. All images are shown at the same magnification. *Bar*, 2.5 μm. *D* represents the quantification of cell size illustrated in *C*; for each condition, the width of randomly chosen cells was measured at the widest point, and the average size is shown together with the S.D.

(Fig. 2*B*). Third, we performed Triton X-114 phase extraction experiments to determine whether Yop1p has the physiochemical properties of an integral hydrophobic membrane protein. In this technique, total cellular proteins are first detergent solubilized at 0 °C. The mixture is then warmed to 30 °C, exploiting the cloud point of Triton X-114 to create two phases that can be separated by gentle centrifugation: a detergent-rich phase containing membrane proteins and an aqueous phase containing hydrophilic proteins. Yop1p partitioned exclusively into the detergent-rich phase (Fig. 2*C*), indicating that it contains hydrophobic domains that anchor it in the lipid bilayer. As a control, fractions were also probed for a known integral membrane protein, Snc1/2p (30), which partitioned into the detergent phase as expected. Taken together, these data show that Yop1p is an integral membrane protein.

Physical Association of Yip1p and Yop1p—To confirm the two-hybrid data, we performed biochemical studies of the Yip1p/Yop1p interaction. For this purpose, we created the strain RCY425, which expresses GST-Yop1p fusion protein under the control of the regulatable $GAL_{1/10}$ promoter and contains a multicopy plasmid expressing Myc_9-Yip1p. We also created an isogenic control strain, RCY428, which expresses GST alone together with Myc_9-Yip1p. Tween 20 detergent-solubilized total lysates were produced from mid-log phase cells grown in galactose and GST fusion proteins were isolated on gluthathione-agarose beads followed by SDS-PAGE and Western blotting to detect any associated Myc-tagged proteins. The results of this experiment are shown in Fig. 3*A*. Myc_9-Yip1p was detected in the GST-Yop1p pull-down but was not detected in the pull-down of GST alone, showing that Myc_9-Yip1p exists in physical association with Yop1p. To rule out any possibility of GST-Yop1p interacting with the Myc epitopes of Myc_9-Yip1p, we repeated the experiment with RCY429, which expresses GST-Yop1p together with Dss4p-Myc_3. In this experiment, the Western blot was first probed with anti-Myc antibody and then reprobed with anti-GST antibody. The results are shown in Fig. 3*B*. Dss4p-Myc_3, Myc_9-Yip1p, and GST-Yop1p are expressed at equivalent levels in the detergent-solubilized lysates. The GST pull-downs reveal that Myc_9-Yip1p associated with GST-Yop1p but Dss4p-Myc_3 did not associate and could not be detected in the pull-down, demonstrating that the biochemical association of Yip1p and Yop1p is specific.

We also repeated the experiment with Yip1p expressed at wild-type levels on a single-copy centromeric plasmid under the control of its own promoter and terminator. For these experiments, Yip1p was tagged with GFP, and lysates were produced with Triton X-100 detergent solubilization. Western blot analysis of the glutathione resin pull-downs (Fig. 3*C*) showed that GFP-Yip1p (RCY509) was specifically isolated with GST-Yop1p, while a control protein, GFP-Ypt7p (RCY508), was not. Western blots of the detergent-solubilized lysates confirmed that GST-Yop1p and the GFP fusion proteins were expressed at equivalent levels in both cases.

To further investigate the relationship between Yip1p and Yop1p, we asked whether the interaction in our pull-down experiments occurred *in vivo* prior to cell lysis or postlysis *in vitro*. For these experiments, we performed the glutathione resin pull-downs on lysates derived from cells coexpressing GST-Yop1p and Myc_9-Yip1p (RCY425) or by combining lysates from individual strains RCY429 (containing GST-Yop1p and Dss4p-Myc_3) or RCY462, which contains Myc_9-Yip1p only. These results can be seen in Fig. 3*D*. We were only able to detect the interaction of Yip1p and Yop1p from cells expressing both proteins simultaneously. These results indicate that Yip1p and Yop1p interact *in vivo*, in a complex that is formed prior to cell lysis.

Overexpression of YOP1 Is Dominant Negative and Can Be Suppressed by Co-overexpression of YIP1—We deleted the entire *YOP1* ORF in a diploid cell that was sporulated and dissected into tetrads to study the phenotype of the haploid-disrupted strain. The *YOP1*Δ haploids were viable, indicating that *YOP1* is dispensable for vegetative growth. Furthermore, a strain carrying the null allele has no apparent growth defect under several conditions commonly used to detect phenotypes in *S. cerevisiae* (31): high temperature (37 °C), low temperature (15 °C), 2 mM caffeine, 2% formamide, high salt (1 M NaCl), and glycerol as carbon source (data not shown). We next examined the phenotype of Yop1p overexpression. For this experiment, we expressed both full-length Yop1p (Yop1p full-length,

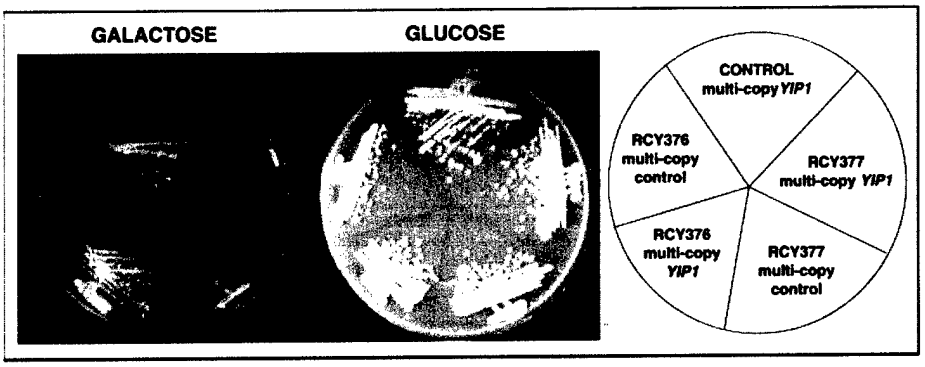


FIG. 5. **Multicopy *YIP1* can rescue the lethality associated with overexpression of full-length Yop1p but not of the Yop1p C-terminal construct that lacks the Yip1p-interacting domain.** RCY376 expresses full-length Yop1p, and RCY377 expresses the Yop1p C terminus in a galactose-dependent manner. The control strain was transformed with the $GAL_{1/10}$ HA-tagged vector only (no insert). Growth of these strains is shown on both glucose and galactose carbon sources with either multicopy *YIP1* or a control multicopy plasmid as indicated.

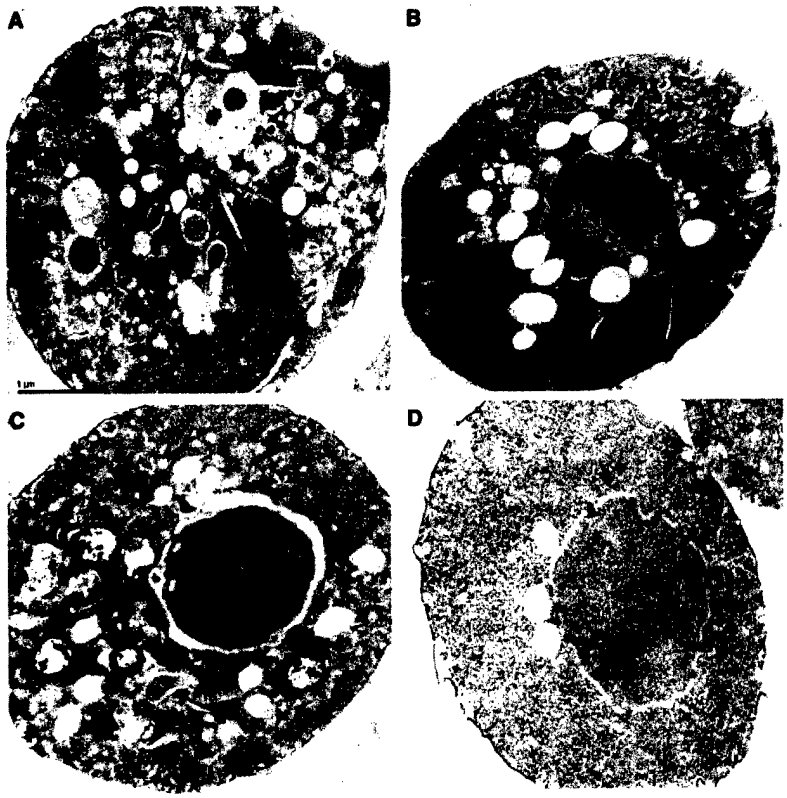


FIG. 6. *A–D*, thin section electron microscopy of dominant negative *YOP1*. RCY376 cells expressing full-length dominant negative Yop1p (*A–C*) and NY605 cells expressing wild-type levels of Yop1p (*D*) were examined by thin section electron microscopy. Representative examples of each strain are shown. *Bar* in *A*, 1 μm. All *panels* are shown at the same magnification.

RCY376) and Yop1p that lacks 17 amino acids at the N terminus identified as the Yip1p-interacting region (Yop1p C terminus, RCY377). Both constructs were expressed in yeast as HA-tagged proteins under the control of the $GAL_{1/10}$ promoter. Immunoblot analysis of the lysates verified that both proteins were expressed in equivalent amounts upon shift of the growth medium to galactose (Fig. 4*A*). Both of these constructs were dominant negative for growth upon overexpression (Fig. 4*B*) but resulted in different morphologies. Overexpression of full-length Yop1p resulted in huge swollen cells of aberrant shape, while overexpression of the Yop1p C-terminal construct gave rise to much smaller cells, similar in shape but considerably smaller than cells harboring a control construct (Fig. 4*C*). To quantitate the observed effect, measurement of cell size was performed (Fig. 4*D*). Wild-type cells have an average width of 5.52 ± 0.90 μm; cells expressing full-length Yop1p have an average width of 6.85 ± 0.97 μm; and cells expressing Yop1p C-terminal construct have an average width of 3.37 ± 0.367 μm.

The dominant negative phenotype of full-length Yop1p overexpression was suppressed by co-overexpression of Yip1p from a multicopy plasmid (Fig. 5). However, multicopy *YIP1* had no effect on the dominant negative phenotype of the Yop1p C-terminal construct, which lacked the Yip1p interaction region. These data further demonstrate that the interaction of Yip1p and Yop1p is a *bona fide* physiological interaction and support the identification of the Yop1p N terminus as the site of Yip1p interaction.

Dominant Negative YOP1 Results in Alteration of Membrane Structures and a Block in Membrane Traffic—To investigate morphological alterations in dominant negative *YOP1* cells in detail, we performed electron microscopy. Cells containing the full-length dominant negative *YOP1* construct and isogenic wild-type cells were grown in galactose before being fixed with and processed for electron microscopy. These results are shown in Fig. 6. Expression of the full-length *YOP1* dominant negative construct resulted in the disappearance of large vacuoles normally seen in wild-type cells and the appearance of smaller and aberrantly shaped compartments filled with darkly stained material (Fig. 6, *A–C*). These cells also contained numerous discontinuous ring-shaped structures; some membrane structures resembled the cup-shaped "Berkeley bodies" known to represent abnormal Golgi structures, while others had pleiomorphic, clublike shapes. In some cells, an accumulation of ER membranes, as judged by their connection to the nuclear envelope, was also observed. Such aberrant membrane structures are not observed in wild-type cells (Fig. 6*D*) and represent

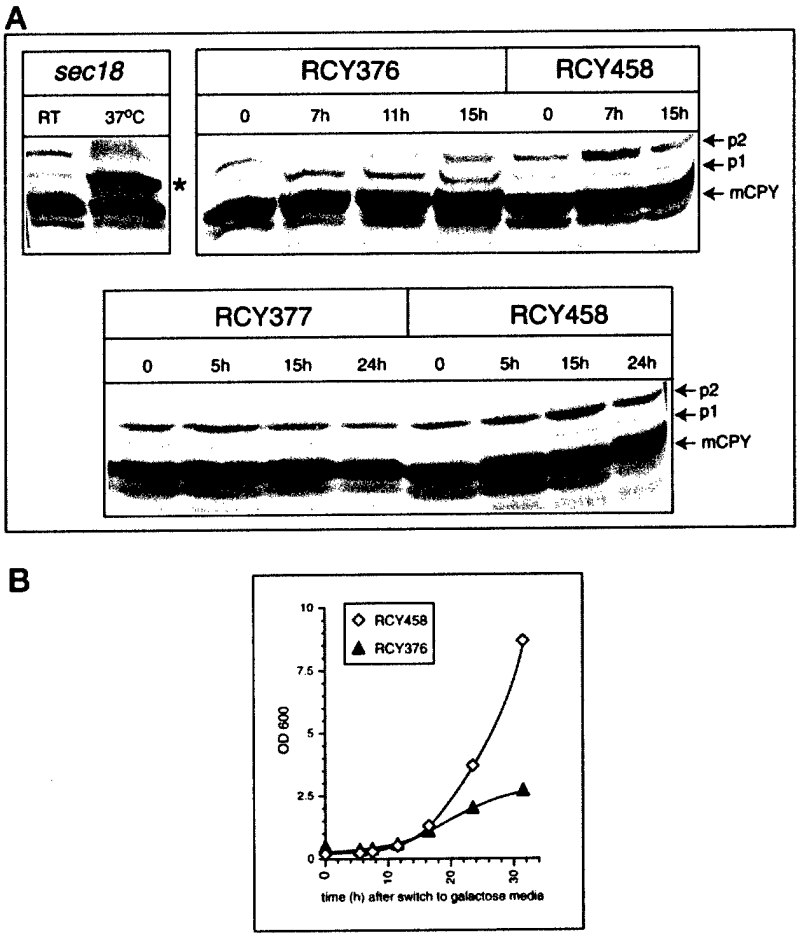


FIG. 7. *A*, CPY immunoblot analysis of cells expressing dominant negative *YOP1* constructs. Shown is immunoblot analysis of total cell lysates for the relative level CPY processing in cells expressing dominant negative constructs containing full-length *YOP1* (RCY376), C-terminal *YOP1* (RCY377), and an isogenic control strain (RCY458). At the time points indicated after the switch from sucrose- to galactose-containing medium, samples were taken and processed for total cell lysates. The *arrows* indicate the relative migration of the p1 (core-glycosylated ER), p2 (Golgi-modified), and m (mature vacuolar) forms of CPY. *sec18* cells are shown at room temperature (permissive temperature) and after shift to 37 °C (restrictive temperature) for 1 h as a control for the migration of the various CPY forms and to provide a positive reference for the accumulation of p1 CPY, marked with *asterisk*. *B*, cell growth of dominant negative *YOP1*. Growth of cells expressing full-length *YOP1* dominant negative construct (RCY376) relative to isogenic wild-type strain (RCY458). Cells were grown to log phase in sucrose-containing selective medium before being switched to galactose-containing medium to induce expression of construct. At various times, as indicated, a turbidity measurement was made as a record of cell growth. Cell concentration was maintained in log phase for the duration of the experiment.

a gross distortion of the normal pathways of membrane traffic in the *YOP1* dominant negative cells.

To investigate the effect of *YOP1* overexpression on membrane traffic, we monitored the steady state level of newly synthesized precursors of the vacuolar protease CPY, the product of the *PRC1* gene. CPY is a soluble vacuole protein that undergoes processing from a core-glycosylated ER form (p1, 67 kDa) to a modified Golgi form (p2, 69 kDa) before being proteolytically cleaved in the vacuole to mature CPY (61 kDa). Using an anti-CPY antibody, we analyzed total cell lysates for the relative levels of the precursor and mature CPY forms under wild-type and YOP1 dominant negative conditions. As a control, we used *sec18* cells shifted to the restrictive temperature at which all stages of membrane traffic are blocked, resulting in the accumulation of the core-glycosylated p1 form of CPY (shown by an *asterisk*). The results are shown in Fig. 7*A*. Cells overexpressing full-length Yop1p show an accumulation of p1 CPY relative to isogenic wild-type cells (RCY458), which is indicative of a block early in exocytosis at the level of the ER. The observed accumulation is the specific result of a block in membrane traffic and does not reflect a generalized disruption of cellular function as the block can be observed within the first 7 h of galactose induction, cell growth rates are not affected until ~16.5 h after galactose induction (Fig. 7*B*). Dominant negative cells overexpressing Yop1p C terminus (RCY377) do not show the same effect, and no accumulation of CPY is observed.

Localization of YOP1—We examined the localization of Yop1p by subcellular fractionation and immunofluorescence. For this purpose, we constructed the strain RCY460, which contains wild-type levels of HA-tagged Yop1p as the sole cellular source of Yop1p. Separation of postnuclear supernatants into P10 (after 10,000 × *g* centrifugation) and P100 and S100 (after 100,000 × *g* centrifugation) followed by immunoblotting with monoclonal anti-HA antibody (12CA5) is shown in Fig. 8*A*. HA-Yop1p fractionates with both light and heavy membranes (P10, P100) but not with cytosol (S100).

By immunofluorescence microscopy, HA-Yop1p appears as a punctate pattern that appears to be at, or near, the periphery of the cell, roughly proportionally distributed between the mother and bud with a greater concentration in the more actively growing region of the cell (Fig. 8*B*). To identify the cellular location of Yop1p, we performed double label immunofluorescence with Sec4p and with GFP-Ypt6p. HA-Yop1p does not localize to the bud tip or at the neck during cytokinesis and can be clearly distinguished from Sec4p immunofluorescence, which is solely concentrated at the leading edge of the cell (Fig. 9*A*). The Yop1p signal partially overlapped with the Ypt6p fluorescence, especially toward the leading edge of the cell, indicating the presence of Yop1p on Golgi membranes (Fig. 9*B*). The HA-Yop1p pattern of expression is identical whether or not the construct is integrated into the genome or maintained as a centromeric plasmid; the expression pattern is also identical in diploids and haploids and on cells grown in glucose, galactose, or glycerol carbon sources (data not shown).

To further examine the subcellular localization of Yop1p and its interactions with Yip1p, we performed confocal microscopy. Cells coexpressing HA-Yop1p and GFP-Yip1p are shown in Fig. 9*C*. Substantial overlap of the Yop1p and Yip1p signal was observed toward the growing edge of the cell, confirming our biochemical data indicating that a physical interaction between Yop1p and Yip1p occurs *in vivo*.

Interaction of Yop1p with Rab Proteins—Since Yip1p is required for secretory pathway function, presumably through its

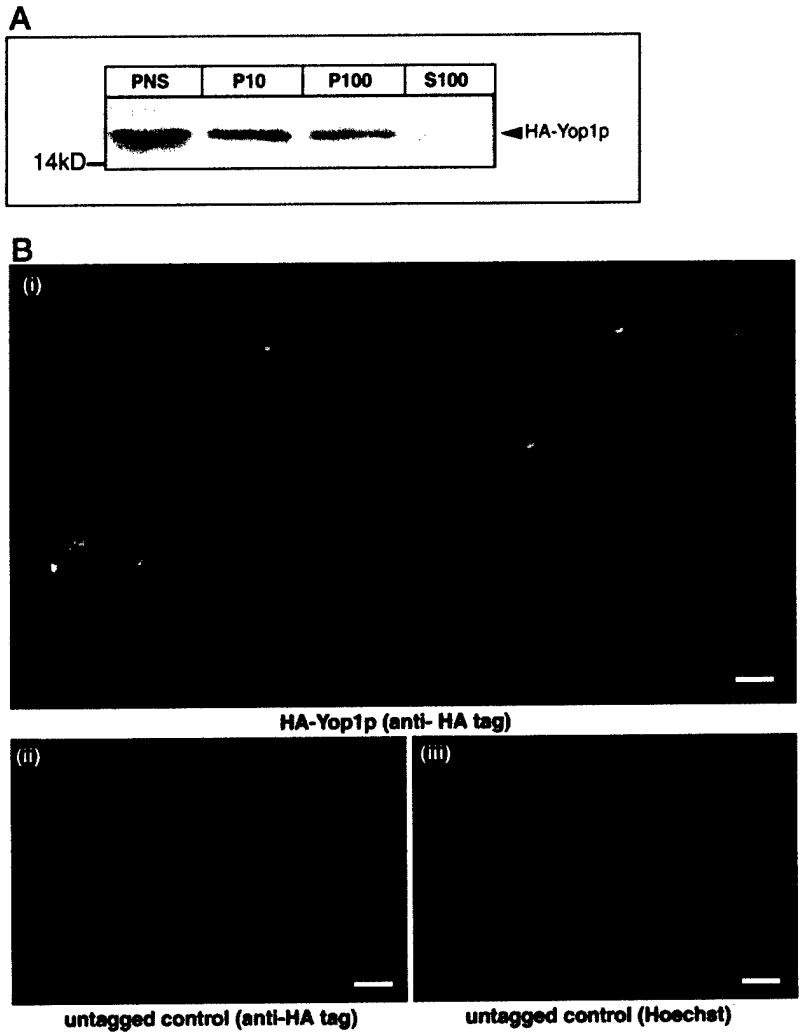


FIG. 8. *A*, intracellular localization of Yop1p. RCY469 cells expressing wild type levels of a single HA epitope-tagged Yop1p as the only source of Yop1p were grown to logarithmic phase, disrupted with glass beads, and subjected to centrifugation at 500 × *g* to remove unbroken cells and cell debris. The PNS was fractionated by differential centrifugation at 10,000 × *g* to give pellet fraction P10 and 100,000 × *g* to yield pellet fraction P100 and supernatant fraction S100. Aliquots of fractions were subjected to SDS-PAGE and Western blot analysis with anti-HA monoclonal antibody. *B*, immunofluorescence localization of Yop1p. RCY469 cells expressing wild type levels of HA-tagged Yop1p as the only source of Yop1p (*A*) and an isogenic control strain, RCY407, expressing the untagged protein (*B* and *C*) were examined by immunofluorescence microscopy. HA-tagged Yop1p was localized with the anti-HA tag antibody Y11 (*A* and *B*). Nuclei were localized in the untagged control by Hoechst 33258 staining (*C*). All *panels* are shown at the same magnification. *Bar*, 5 μm.

effects on Rab proteins, we sought to investigate any possible interaction of Yop1p with Rab proteins. There are 11 Rab protein family members in *S. cerevisiae*; however, some of these represent closely related isoforms (*e.g.* Vps21p, Ypt52p, and Ypt53p). To gain as complete an insight as possible, we made GST fusions with several Rab proteins encompassing representatives from each subset. These proteins were expressed in cells behind the galactose promoter and were tested for interaction by coprecipitation with Yop1p, which was tagged with a single HA epitope and also expressed behind the galactose promoter. The results of this analysis are shown in Fig. 10*A*. HA-Yop1p did not coprecipitate with GST alone but was able to precipitate with GST fused to the Rab proteins Ypt52p, Sec4p, Ypt6p, and Ypt7p. The observed interaction was stable in buffers containing 0.5% Tween 20; however, it could not be observed in 0.5% Triton X-100 containing buffers (data not shown). No interaction was observed with a Ypt1p construct lacking its C-terminal cysteines, which are the sites of prenylation. The interactions between Yop1p and Rab proteins are unlikely to be real *in vivo* interactions; otherwise, the steady-state localization of Yop1p would probably be more universally distributed among subcellular membranes. It is more likely that the observed interactions reflect a generalized biochemical ability of Yop1p to interact with a common determinant of fully post-translationally modified Rab proteins, an interaction that can be revealed by overexpressing both proteins and performing coprecipitation assays as shown in Fig. 10*A*. To reveal which Rab protein may be important for Yop1p action *in vivo*, we performed suppression analysis of the *YOP1* dominant negative constructs with multicopy plasmids encoding all 11 Rab proteins of *S. cerevisiae*. The full-length *YOP1* dominant negative construct, while able to be suppressed by multicopy *YIP1* (Fig. 5), could not be suppressed by overexpression of any of the genes encoding the yeast Rab proteins (data not shown). However, multicopy *YPT6* was able to suppress the dominant negative phenotype of the *YOP1* C-terminal construct. The suppression of the *YOP1* C-terminal construct (RCY377) by 2μ *YPT6* together with *YPT7* and *DSS4* as a comparison is shown in Fig. 10*B*. *YPT6* was the only Rab gene capable of causing *in vivo* suppression of RCY377; no other Rab gene tested (*SEC4*, *YPT1*, *YPT31*, *YPT32*, *VPS21*, *YPT52*, *YPT53*, *YPT10*, *YPT11*, and *YPT7*; data not shown except for *YPT7*, Fig. 10*B*), was able to restore growth.

DISCUSSION

We have isolated *YOP1* as a novel *YIP1*-interacting clone in a yeast two-hybrid screen of a yeast genomic library. Yop1p and Yip1p are both integral membrane proteins. The interaction of a membrane protein in the two-hybrid system is perhaps surprising and worthy of comment. Yip1p is not alone in this regard; other membrane proteins have also been shown to functionally interact in such a system (29). Although some membrane proteins clearly cannot maintain their native structure and functional interactions in the two-hybrid system, there are at least two factors that might indicate whether or not the two-hybrid system will be useful for any given protein. (i) The GAL4 system contains a strong nuclear localization signal and so may dominate over other localization signals

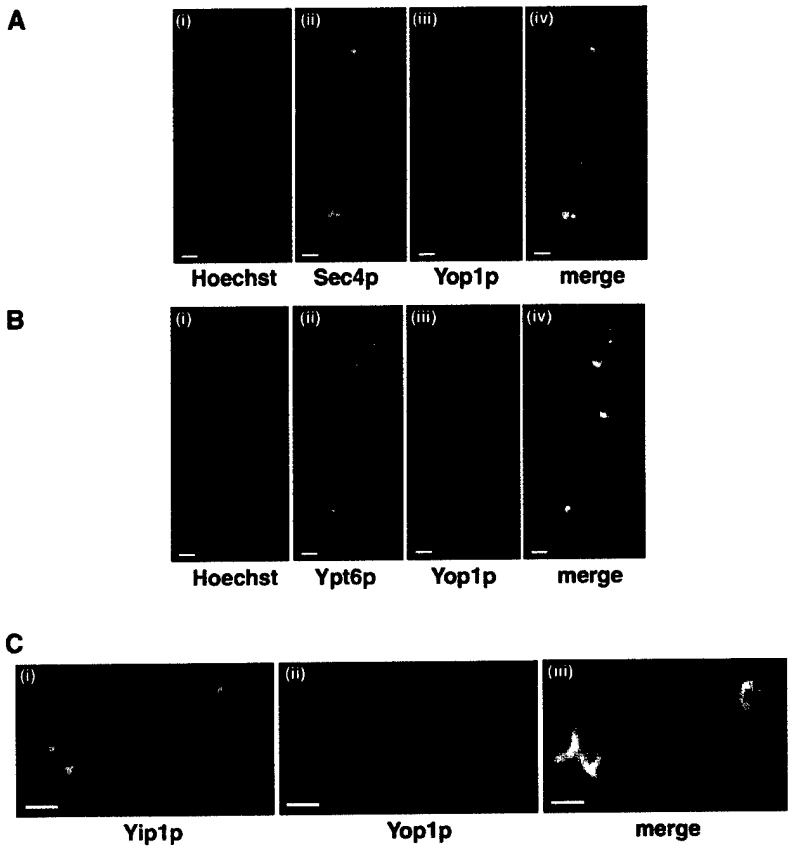


FIG. 9. *A–B*, double label immunofluorescence microscopy of Yop1p with Sec4p and Ypt6p. RCY469 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of either GFP-tagged Sec4p or Ypt6p were examined by double label immunofluorescence microscopy. Cells were labeled with the anti-HA tag antibody Y11 to visualize HA-Yop1p (*A* and *B* (*i*)) and with anti-Sec4p (*A* (*ii*)) or anti-GFP (*B* (*ii*)). Nuclei were counterstained with the DNA stain Hoechst 33258 (*A* and *B* (*iii*)). A merge of all three channels is shown in *A* and *B* (*iv*). Note that under the processing conditions for immunofluorescence, there was no interference from the intrinsic GFP fluorescence. *C*, double label confocal immunofluorescence microscopy of Yop1p with Yip1p. RCY496 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of GFP-tagged Yip1p were examined by double label confocal microscopy. Cells were labeled with anti-HA tag antibody (*i*) and with anti-GFP antibody (*ii*). A merge of both channels is shown in *iii*.

present in the two-hybrid construct and be a better system for this purpose than a system that relies on passive diffusion to enter the nucleus (32). (ii) *S. cerevisiae* is probably more capable of correctly folding endogenous yeast proteins rather than proteins from other organisms. In addition, membrane channels have been observed in the nucleus (33), and some viruses acquire membranes in the nucleus (34), indicating that the ultrastructure of the nucleus may be more complex than originally thought.

We have identified two functional domains of Yop1p that act in a dominant manner to inhibit cell growth upon overexpression. The first domain consists of the cytosolic N terminus of Yop1p that corresponds to the first exon of the *YOP1* gene. The second domain comprises the C terminus of the molecule that is mainly hydrophobic and corresponds to the second genomic exon. Overexpression of full-length Yop1p leads to inhibition of cell growth and a phenotype of enlarged cells that accumulate internal membrane structures. This phenotype can be suppressed by co-overexpression of Yip1p. Presumably, Yip1p is able to suppress the toxic effect of Yop1p by sequestration. These data would suggest that *YOP1* overexpression inhibits cell growth by inhibiting the function of Yip1p, since Yip1p is an essential gene required for secretion. Consistent with this interpretation is the phenotype of *YOP1* full-length overexpression, which results in the accumulation of membrane structures and an accumulation of the ER core-glycosylated form of CPY, indicating a block in ER to Golgi traffic. Recently, a Yip1p homolog, Yif1p, has been identified (35) that appears to act similarly to Yip1p in blocking ER to Golgi transport. Although nothing is known about the precise function of Yip1p, the identification of Yif1p and Yop1p as Yip1p binding partners suggests that Yip1p may be involved in several different Rab-mediated events through a combinatorial assortment with different binding partners.

The phenotype of the *YOP1* C-terminal construct is distinct from that of the full-length construct. Yip1p co-overexpression cannot suppress the dominant negative effect of Yop1p C terminus overexpression. The Yop1p-C-terminal construct lacks the domain that is both necessary and sufficient for Yip1p interaction by two-hybrid analysis. The mechanism by which this construct inhibits growth cannot be directly via an inhibition of Yip1p function. One clue may be provided by the fact that *YPT6* can suppress the dominant negative *YOP1* C-terminal construct but not that of the full-length construct, indicating that the action of Yop1p is intimately connected to Rab function. This finding further underscores our results, demonstrating that Yop1p can be specifically coprecipitated with Rab proteins in cellular lysates. Since Yop1p shows a restricted sub-cellular localization, we hypothesize that the biochemical interaction of Yop1p with Rab proteins is limited *in vivo*, possibly only to *YPT6*, which our suppression analysis demonstrates to interact genetically with *YOP1*. Consistent with this interpretation are our data demonstrating that the steady-state immunofluorescent localization of Yop1p and Ypt6p shows overlap *in vivo*.

The two domains of Yop1p may act antagonistically, or perhaps the exposed Yop1p N terminus may constitute a signaling domain that acts in a dose-dependent manner to negatively regulate membrane traffic. There is a growing appreciation that many proteins involved in the regulation of intracellular membrane traffic may act as signal transducers that coordinate membrane traffic with other cellular events (36–38). Different branches of the Ras superfamily are ideally placed to coordinate such cross-talk, and our data indicate that *YOP1* and possibly its human homolog *TB2* may also play a role in the regulation of cell growth through its facilitation of membrane traffic. Our genetic data suggest that *YOP1* is a recessive gene that negatively regulates cell growth. Deletion of *YOP1* has no apparent effect on cell viability, and full-length and C-terminal *YOP1* constructs

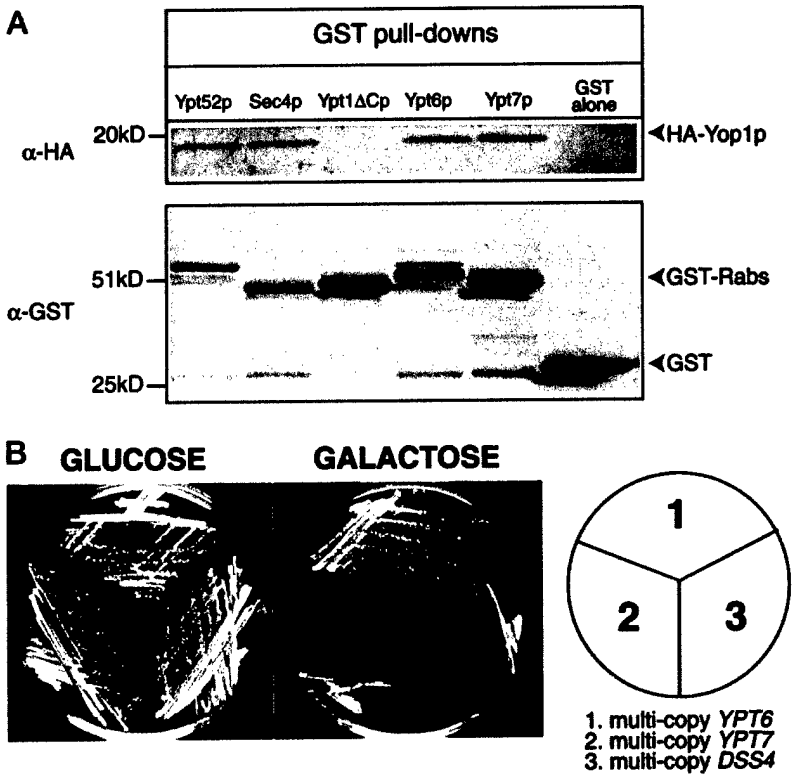


FIG. 10. *A*, coprecipitation of Yop1p with Rab proteins. Lysates were prepared from cells expressing either GST alone or various GST-Rab constructs as indicated, together with HA-tagged-Yop1p. Detergent-solubilized lysates containing 0.5% Tween 20 were incubated with GST beads for 30 min at 4 °C as described under "Experimental Procedures." After four washes, the bead-bound material was subject to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with both polyclonal anti-GST (1:800) to detect the bead-bound GST fusion proteins and monoclonal 12CA5 to detect any associated HA-Yop1p. Relevant protein marker sizes are indicated. All constructs were under the control of the $GAL_{1/10}$ promoter and were expressed by inducing with galactose for ~8 h. HA-Yop1p was detected associated with Ypt52p, Ypt6p, Sec4p, and Ypt7p GST fusion proteins but not on GST alone or Ypt1p lacking its C-terminal cysteines. *B*, growth of dominant negative YOP1 C-terminal construct (RCY377) with various plasmids. RCY377 expresses the Yop1p C terminus in a galactose-dependent manner. The control strain was transformed with the $GAL_{1/10}$ HA-tagged vector only (no insert). Growth of these strains is shown on both glucose and galactose carbon sources with either multicopy *YPT6*, *YPT7*, or a control multicopy plasmid as indicated.

possess a dose-dependent growth inhibitory effect.

Sequence comparison revealed that Yop1p is homologous to the human *TB2* gene with sequence similarities throughout the protein. The amino acid sequence conservation of Yop1p across species clearly points to its functional importance, and an interesting finding is that *TB2* is a human familial adenomatous polyposis locus (39) gene (40), adjacent to the tumor suppressor genes *MCC* and *APC* (41). *TB2* encodes a 197 amino acid polypeptide (1). The deduced amino acid sequence predicts that Yop1p contains at least two extensive membrane-spanning segments. The human *TB2* gene also contains a similar size and type of membrane-spanning segments and would be predicted to have the same topology. This similarity raises the possibility that Yop1p and TB2 may share a common function in mediating vesicular transport. It is now clear that the machinery and mechanisms of membrane traffic share much in common between yeast and higher eukaryotes (42). For example, the complex observed between Sec9p, Sso1/2p, and Snc1/2p, which is required for exocytosis in yeast, is the structural and functional counterpart of the neuronal SNARE complex (30). Rabs are also extremely well conserved over evolution. In some cases, yeast and mammalian Rab proteins are functionally interchangeable. For example, Vps21p/Ypt51p, a homolog of mammalian Rab5, is also required at an early step in endocytic traffic (43). Remarkably, Ypt51p expression in animal cells not only localizes to Rab5-positive early endosomes but also stimulates endocytosis (44). This latter fact indicates that the machinery involved in mediating Rab protein function is probably conserved across diverse species. Our data indicate that Yop1p, probably in conjunction with Yip1p, acts to facilitate a Rab-mediated event in membrane traffic. It remains to be demonstrated whether TB2 has a role in membrane transport.

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Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors

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Abstract The Rab GTPases are key regulators of membrane traffic. Yip1p is a membrane protein of unknown function that has been reported to interact with the Rabs Ypt1p and Ypt31p. In this study we identify Yif1p, and two unknown open reading frames, Ygl198p and Ygl161p, which we term Yip4p and Yip5p, as Yip1p-related sequences. We demonstrate that the Yip1p-related proteins possess several features: (i) they have a common overall domain topology, (ii) they are capable of biochemical interaction with a variety of Rab proteins in a manner dependent on C-terminal prenylation, and (iii) they share an ability to physically associate with other members of the YIP1 family.

Key words: Rab; YIP1; YIP4; YIP5; YGL198W; YGL161C

1. Introduction

Rab GTPases form the largest branch of small GTPases in the Ras superfamily and are found in all eukaryotic organisms [1]. Rab proteins perform essential functions in different membrane transport pathways of the cell such as vesicle biogenesis [2], targeting and fusion of membrane-bound containers [3], and the association of organelles with motor proteins [4].

Like other members of the Ras superfamily, the intrinsic interconversion rates between the GDP- and GTP-bound forms of the protein are regulated by accessory factors such as guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In addition to their cycle of nucleotide binding and hydrolysis, Rab proteins also undergo cycles of membrane association and dissociation. Rab proteins stably attach to membranes by virtue of their post-translational prenylation modification: the attachment of two C20 geranylgeranyl groups onto C-terminal cysteines of the protein [5]. The Rab protein can be removed from the membrane through the action of Rab-GDP dissociation inhibitor (GDI). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety [6]. The heterodimer of GDP/Rab-GDI enables the Rab protein to be recycled through the cytosol back onto membranes for subsequent rounds of transport. The membrane recruitment reaction of Rabs is highly specific, each organelle of the secretory and endocytic pathways is found to associate with a particular Rab protein(s).

To date, many of the Rab interacting proteins that have been identified are soluble factors whose activity can be assigned to defined classes such as effectors, GEFs, GAPs etc. based on their ability to modulate the Rab GTPase cycle. Recently, several Rab interacting membrane proteins have been identified. These include Yip1p, PRA1, rab5ip and Yop1p [7–10]. The existence of these proteins raises the exciting possibility that they are involved in regulating Rab function on membranes or perhaps modulate the association of Rab proteins with membranes. In this study, we have focused on one of this class of membrane proteins, Yip1p. Using Yip1p as a departure point we have identified YIP1-related sequences and demonstrate that the proteins encoded by these sequences have common characteristics and constitute a protein family. Because Yip1p is the founder member or prototype for this family we have termed it the YIP1 family. For small membrane proteins such as Yip1p, identification of homologs cannot be confidently predicted based on primary sequence comparison alone. This is due to the fact that large stretches of the protein consist of hydrophobic residues, reducing the complexity necessary for successful database mining. Our results define three additional criteria for a Yip1p-related protein. These criteria are a common domain topology, the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and the ability to associate physically with other Yip1p family members. We demonstrate that Yif1p, and two unknown open reading frames (ORFs), YGL198W and YGL161C, share these features and qualify as YIP1 family members: we have termed these ORFs Yip4p and Yip5p respectively. The YIP1-related proteins are found across eukaryotes and YIP1 family members have both overlapping and distinct functions.

2. Materials and methods

2.1. Yeast strains and media

The *Saccharomyces cerevisiae* strains used in these studies are listed in Table 1. All yeast strains were manipulated as described in [11].

2.2. Yeast two-hybrid (Y2H) assay

The ORF sequences were subcloned into pAS1-CYH2 or pAS2-1 for 'bait' and and pACTII or pACT2 for 'prey' constructs respectively as listed in Table 2. pRC187 and pRC188 are two independent bait constructs which contain Yip1p. pRC1466 and pRC1467 are two in-

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Abbreviations: GDI, GDP dissociation inhibitor; GST, glutathione *S*-transferase; GAP, GTPase activating factor; GEF, guanine nucleotide exchange factor; Y2H, yeast two-hybrid; MBP, maltose binding protein; GFP, green fluorescent protein; 5-FOA, fluoroorotic acid

0014-5793/02/$22.00
PII: S0014-5793(02)02442-0

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
RCY427	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1-10}$*GST*	This laboratory [13]
RCY442	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT7*	This laboratory [13]
RCY539	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1*	This laboratory [13]
RCY693	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT10*	This laboratory [13]
RCY694	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT11*	This laboratory [13]
RCY695	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT31*	This laboratory [13]
RCY696	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT32*	This laboratory [13]
RCY697	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT52*	This laboratory [13]
RCY698	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT6*	This laboratory [13]
RCY699	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-SEC4*	This laboratory [13]
RCY700	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT1ΔC*	This laboratory [13]
RCY701	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT1*	This laboratory [13]
RCY765	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST* [pRC1054]	This study
RCY850	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1* [pRC1053]	This study
RCY780	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST* [pRC1047]	This study
RCY851	*MATa ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1* [pRC1047]	This study
RCY873	*MATa ura3-52 leu2-3,112* [$P_{GAL1/10}$GST-Yip4p CEN *LEU2* pRC1578] [MBP-Yip4p pRS426 pRC1053]	This study
RCY881	*MATa ura3-52 leu2-3,112* [$P_{GAL1/10}$GST-Yif1p CEN *LEU2* pRC1579] [MBP-Yip4p pRS426 pRC1053]	This study
RCY1354	*MATa ura3-52 leu2-Δ1 lys2-801 his3Δ200 ade2-101 trp1-Δ63 YIP1ΔHIS* [YCP50 *YIP1* pRC1245]	This study
Y190	*MATa gal4Δ gal80Δ trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL10→LacZ, LYS2::GAL10→HIS3 cyh*^{R}	Elledge laboratory

dependent prey constructs which contain Ygl161p (Yip5p). The yeast strain Y190 was used to assay for interacting constructs [12]. Due to batch variability in Y2H assays each complete experiment was carried out in a complete set which included positive and negative controls. We also commonly observed variability in the Y2H system between two otherwise identical constructs and so two independently generated constructs were used to confirm interactions observed in our experiments. Pairs of plasmids were cotransformed into the yeast strain and

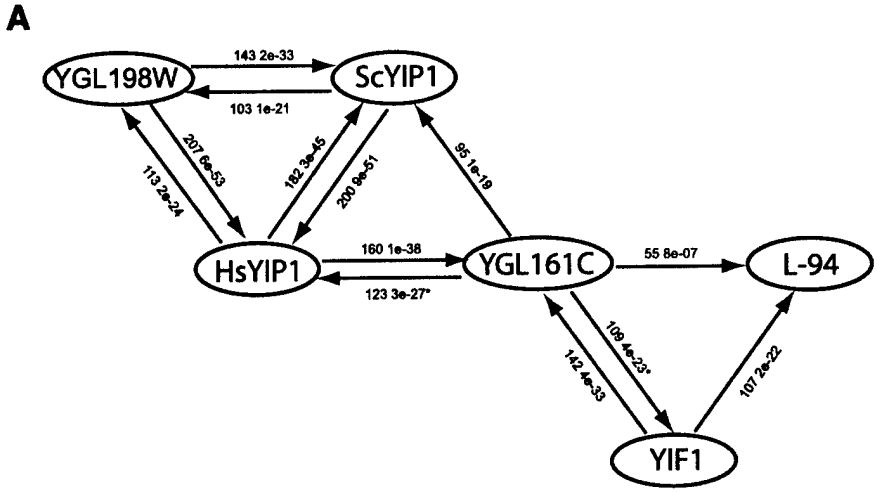
Table 2
Plasmids used in this study

Name	Relevant features	Source
pRC38	pAS1-CYH2 Gal4-DNA binding domain Ypt7p fusion	This study
pRC22	pAS1-CYH2 Gal4-DNA binding domain Yif1p fusion	This study
pRC27	pAS1-CYH2 Gal4-DNA binding domain Ypt11p fusion	This study
pRC34	pAS1-CYH2 Gal4-DNA binding domain Ypt52p fusion	This study
pRC33	pAS1-CYH2 Gal4-DNA binding domain Ypt53p fusion	This study
pRC804	pAS1-CYH2 Gal4-DNA binding domain Ypt1p fusion	This study
pRC805	pAS1-CYH2 Gal4-DNA binding domain Ypt51p fusion	This study
pRC966	pAS1-CYH2 Gal4-DNA binding domain Sec4p fusion	Novick laboratory [27]
pRC29	pAS1-CYH2 Gal4-DNA binding domain Ypt31p fusion	This study
pRC31	pAS1-CYH2 Gal4-DNA binding domain Ypt32p fusion	This study
pRC25	pAS1-CYH2 Gal4-DNA binding domain Ypt10p fusion	This study
pRC1253	pAS1-CYH2 Gal4-DNA binding domain Dss4p fusion	Novick laboratory [27]
pRC225	pAS2-1 Gal4-DNA binding domain human Yip1p fusion	This study
pRC181	pAS1-CYH2 Gal4-DNA binding domain Yip4p fusion	This study
pRC977	pAS1-CYH2 Gal4-DNA binding domain Sec4ΔCp (Sec4p lacking C-terminal cysteines) fusion	Novick laboratory [27]
pRC187/pRC188	pAS1-CYH2 Gal4-DNA binding domain Yip1p fusion	This study
pRC957	pACTII Gal4-DNA activation domain Yip1p fusion	This study
pRC42	pACTII Gal4-DNA activation domain Yif1p fusion	This study
pRC44	pACTII Gal4-DNA activation domain Yip4p fusion	This study
pRC1464	pACTII Gal4-DNA activation domain Gdi1p fusion	Novick laboratory [27]
pRC1466/pRC1477	pACTII Gal4-DNA activation domain Yip5p fusion	This study
pRC1047	MBP tagged Yif1p *URA3* 2μm (pRS426)	This study
pRC1049	MBP tagged Yip1p *URA3* 2μm (pRS426)	This study
pRC1053/pRC1054	MBP tagged Yip4p *URA3* 2μm (pRS426)	This study
pRC337	*LEU2* INT $GAL_{1/10}$ GST (pRS305)	This laboratory [13]
pRC696	*LEU2* INT $GAL_{1/10}$ GST-Ypt10p (pRS305)	This laboratory [13]
pRC697	*LEU2* INT $GAL_{1/10}$ GST-Ypt11p (pRS305)	This laboratory [13]
pRC698	*LEU2* INT $GAL_{1/10}$ GST-Ypt31p (pRS305)	This laboratory [13]
pRC699	*LEU2* INT $GAL_{1/10}$ GST-Ypt32p (pRS305)	This laboratory [13]
pRC700	*LEU2* INT $GAL_{1/10}$ GST-Ypt52p (pRS305)	This laboratory [13]
pRC701	*LEU2* INT $GAL_{1/10}$ GST-Ypt6p (pRS305)	This laboratory [13]
pRC702	*LEU2* INT $GAL_{1/10}$ GST-Sec4p (pRS305)	This laboratory [13]
pRC711	*LEU2* INT $GAL_{1/10}$ GST-Ypt1ΔC (pRS305)	This laboratory [13]
pRC1016	*LEU2* INT $GAL_{1/10}$ GST-Ypt1p (pRS305)	This laboratory [13]
pRC726	*LEU2* INT $GAL_{1/10}$ GST-Yip1p (pRS305)	This laboratory [13]
pRC1245	YCP50 containing *YIP1* with endogenous 5′ and 3′ UTR	This study
pRC1578	*LEU2* CEN $GAL_{1/10}$ GST-Yip4p (pRS315)	This study
pRC1579	*LEU2* CEN $GAL_{1/10}$ GST-Yif1p (pRS315)	This study

at least 30 independent colonies were assayed for β-galactosidase activity. β-Galactosidase activity was determined with the chromogenic substrate X-gal using a Macintosh computer-based imaging analysis with CanoScan N670U using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/)

2.3. Co-precipitation experiments

Rab proteins as indicated were expressed as glutathione *S*-transferase (GST) fusion proteins under the control of the $GAL_{1/10}$ promoter in yeast. These strains contain a plasmid expressing either maltose binding protein (MBP)-tagged Yip1p, Yif1p or Yip4p. The experimental protocol was as described in [13]. Strains used for pull-down experiments were grown overnight in 50 ml of selective medium containing galactose as carbon source (SGal) to an absorbance of ~0.7 A_{600}. Cells were harvested by centrifugation at 4°C and washed in 1 ml of ice-cold buffer (10 mM Tris pH 7.5, 10 mm NaN_3). Cell pellets were resuspended in 100 μl of ice cold lysis buffer (20 mM KPi pH 7.5, 80 mM KCl, 1 mM EGTA, 2% glycerol, 0.8% Tween 20) containing protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 10μg/ml pepstatin A) before lysis with glass beads. A total detergent-solubilized extract was generated by incubating lysates



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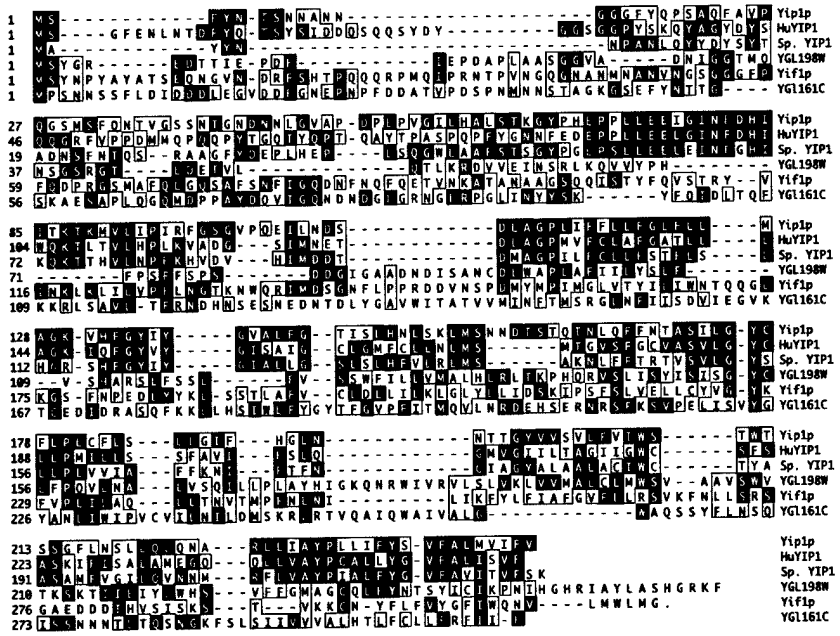


Fig. 1. A: PSI-BLAST identification of related YIP1 sequences. PSI-BLASTP 2.2.1 was performed on each protein sequence indicated. Relationships identified are indicated using lines whose directionality points from the query sequence towards the identified sequence. Analysis was carried out using a threshold value of $p = 0.01$ (p value = 0.1 indicated with asterisk) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772993 sequences. The complete set of statistical values for these sequence relationships is given in Table 3. B: Alignment of Yip1p with *S. cerevisiae* and human homologs. Sequence of Yip1p and comparison with full length cDNAs from *S. pombe* (SpYIP1), human YIP1 (HsYIP1), Yif1p and the novel *S. cerevisiae* ORFs YGL198W and YGL161C. The sequences were aligned in MegAlign (DNASTAR) using Clustal analysis [25] with a gap length penalty of 10. Amino acid residues are numbered according to the protein sequence. The shaded residues exactly match the consensus sequence, the boxed residues are standard functional groupings [26] of acidic (DE), basic (HKR), hydrophobic (AFILMPVW), and polar (CGNQSTY) residues. Sequence identity values are given in Table 4.

with an additional 1 ml of lysis buffer for 10 min at 4°C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microfuge for 5 min at 13000 rpm. Samples were incubated with rocking for 30 min at 4°C with 20 μl of amylose resin (New England Biolabs). The bead-bound material was washed four times with lysis buffer. Similar procedures were followed for GST pull-downs except glutathione *S*-Sepharose resin (Pharmacia) was used to isolate the GST-tagged proteins. Proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were analyzed by 10% SDS–PAGE gel electrophoresis and Western blotting with anti-GST antibody to detect the presence of the GST-tagged Rab proteins (for these purposes the anti-green fluorescent protein (GFP) antibody Santa Cruz Cat. No. SC-8334, lot G030 was used, this antibody recognized GST in Western blots with far higher avidity than GFP). Anti-MBP antibody (gift of G.R. Whittaker) was used at 1:6000 to detect MBP-tagged proteins. Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories) were added in blocking buffer, followed by washing and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$).

Protein expression under the control of the $GAL_{1/10}$ promoter was achieved by subcloning the ORF containing the Rab protein in frame with GST into the vector pRC337. These constructs (Table 2) were linearized with a restriction enzyme and integrated into the genome at the *LEU2* locus. Expression of a GST fusion protein of the correct molecular weight was determined by growing the cells in media containing 2% galactose as a carbon source. The plasmid containing MBP-tagged Yip1p (pRC1047) was constructed using polymerase chain reaction to insert a MBP tag cassette immediately after the initiating methionine in order to express the fusion protein under the control of the endogenous promoter and terminator in the yeast vector pRS426.

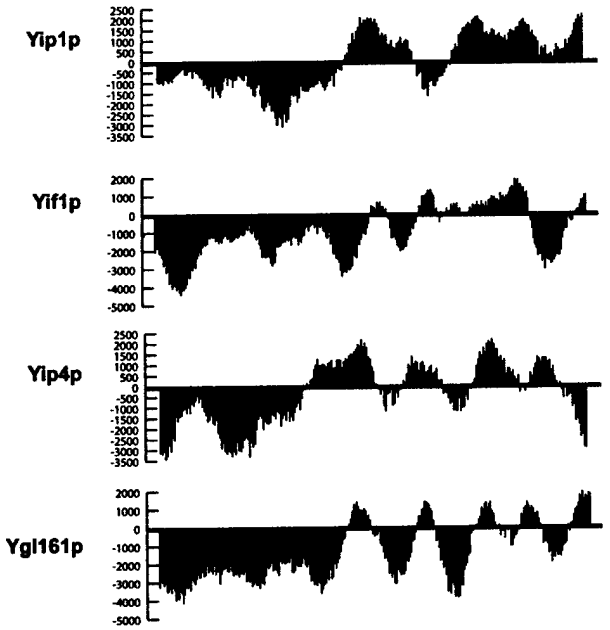


Fig. 2. TMpred plot of Yip1p, Yif1p, Ygl198p and Ygl161p. The TMpred plots for Yip1p, Yif1p, Ygl198p (Yip4p), and Ygl161p (Yip5p) were generated using the program TMpred with a 17 residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix. The TMpred plot shows the relative location of the hydrophobic/hydrophilic segments of the protein. Sequence data indicate a cytoplasmically oriented N-terminus and a hydrophobic C-terminal domain with several potential membrane-spanning/insertion segments.

Table 3
PSI-BLAST score values amongst YIP1-related proteins

Query	Subject sequence													
	ScYIP1		YGL198W		YGL161C		YIF1		HsYIP1		SpYIP1		L-94	
	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value
ScYIP1	221	3e-57	103	1e-21	34	1.8	36	0.24	200	9e-51	184	4e-46	NF	
YGL198W	142	2e-33	300	7e-81	NF		NF		207	6e-53	160	1e-38	NF	
YGL161C	98	1e-19	NF		272	2e-72	109	4e-23	131	7e-30	91	1e-17	55	8e-07
YIF1	NF		NF		NF		320	7e-87	NF		NF		106	3e-22
HsYIP1	182	3e-45	113	2e-24	160	1e-38	33	2.2	296	1e-79	201	6e-51	NF	
SpYIP1	172	2e-42	114	5e-25	44	0.001	NF		216	9e-56	218	2e-56	NF	

PSI-BLASTP 2.2.1 was used to identify YIP1-related proteins. Analysis was carried out using a threshold value of $p = 0.01$ (except for YGL161C for which a p value = 0.1) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772993 sequences. Identified sequences converged after five iterations (YGL198W), six iterations (HsYIP1, ScYIP1), seven iterations (YIF1, YGL161C, SpYIP1). NF, not found.

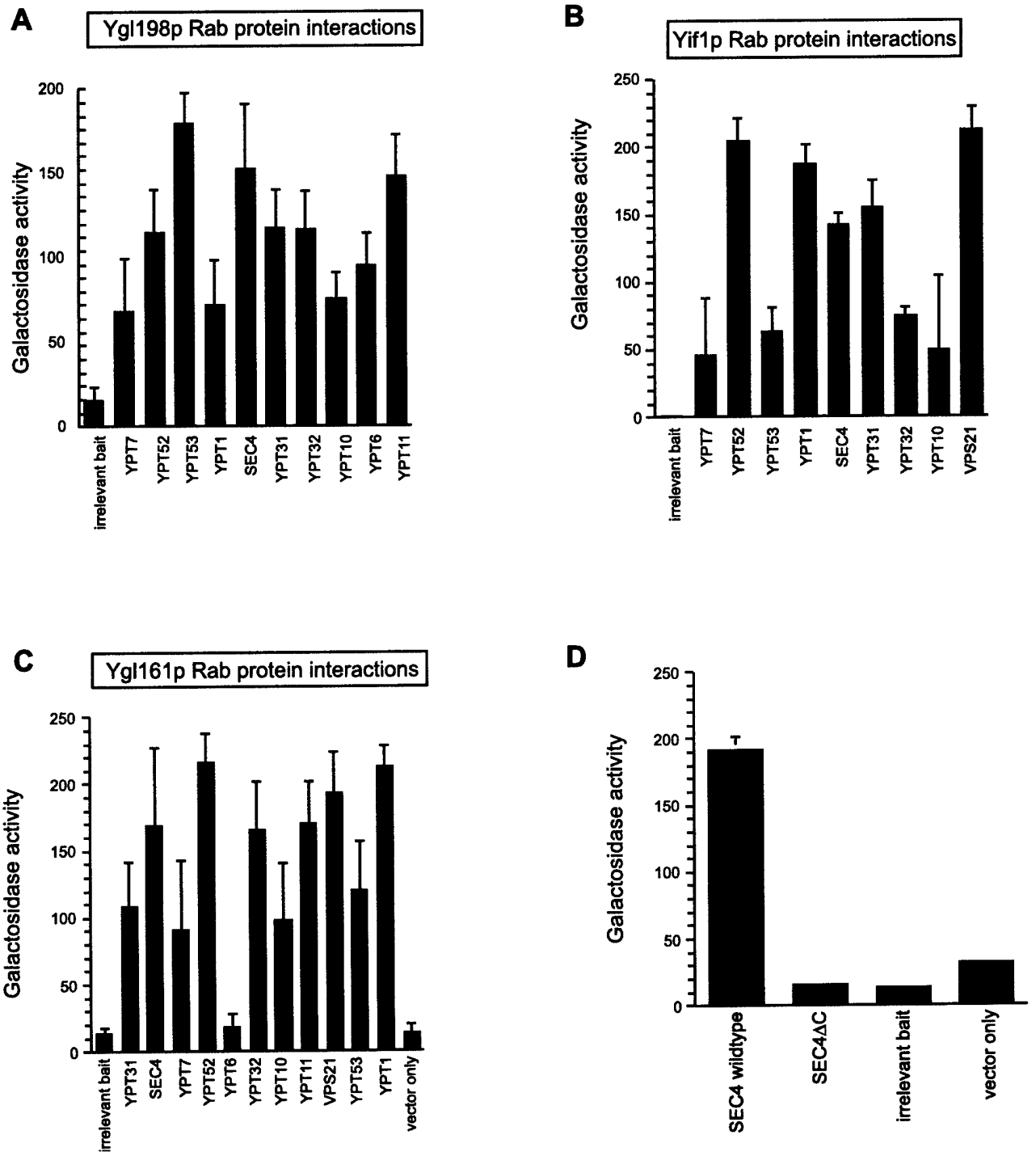


Fig. 3. Y2H interactions of Rab proteins with Yip1p family members. Pairs of constructs were coexpressed in the reporter strain Y190 and β-galactosidase activity (arbitrary units) in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The Rab protein bait constructs as indicated on the *x*-axis were tested against prey constructs of Ygl198p (A), Yif1p (B), and Ygl161p (C). D: Ygl161p prey construct tested against the Rab protein Sec4p with and without the C-terminal cysteines. A construct expressing Dss4p (pRC1253) was used as an irrelevant bait control.

3. Results and discussion

3.1. A family of Yip1p-related proteins

We used PSI-BLAST [14] with $p=0.01$ and the BLOSUM62 matrix to identify Yip1p- and HsYip1p-related proteins. This analysis revealed one known ORF (*YIF1*) and one unknown ORF in *S. cerevisiae* (*YGL198W*), unknown ORF SPCC61.04c in *Schizosaccharomyces pombe*, together with numerous expressed sequence tag (EST) fragments from different species, indicating that *YIP1* is part of a gene family conserved among eukaryotes (Fig. 1A and Table 3). Using

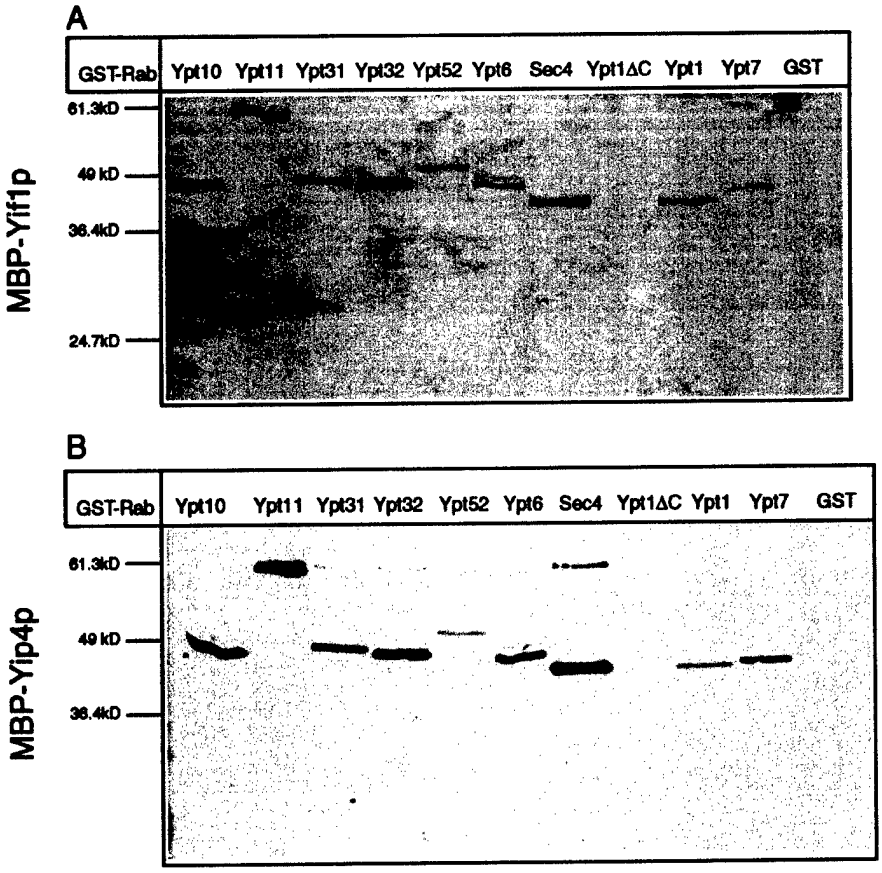


Fig. 4. Co-precipitation of Yip1p-related proteins with Rabs. The panel shows glutathione-resin pull-downs from yeast cells expressing various GST-Rab constructs as indicated. Although the level of expression of proteins in this system is not as high as recombinant expression, it was necessary to use a eukaryotic system due to the dependence of the interaction on correct C-terminal prenylation of the Rab protein. Lysates were prepared from cells expressing either GST alone or various GST-Rab constructs as indicated, together with MBP-tagged Yif1p, or Yip4p. Detergent-solubilized lysates containing 0.5% Tween 20 were incubated with amylose resin for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS–PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (dilution 1:800) to detect the bead-bound GST Rab fusion proteins. Relevant protein marker sizes are indicated. All Rab constructs were under the control of the $GAL_{1/10}$ promoter and were expressed by inducing with galactose for ~8 h.

Yif1p as the query for a PSI-BLAST search with the same parameters yielded the sequences L1–94 and an unknown ORF, YGL161C, with a convergence after seven iterations. We used the identified ESTs to generate a full length clone for human YIP1 which sequencing revealed was 38.3% identical to that of yeast *YIP1*. This sequence is identical to YIP1A, a human protein that has been reported to localize to endoplasmic reticulum exit sites [15] and also to the smooth muscle cell-associated protein-5 (accession number BAB20270). L1–94 is a partial sequence identified as a putative Rab5-interacting protein from human HeLa cells [16]. Yif1p is a protein previously isolated as a Yip1p interacting factor [17], although its homology to Yip1p was not identified. YGL198W and YGL161C are novel ORFs of unknown function in the *S. cerevisiae* database. The PSI-BLAST score (bits) and *E* values showing the relationships amongst these proteins are shown in Table 3 and a family alignment of the YIP1-related proteins is shown in Fig. 1B. This alignment includes only complete ORFs, L1–94 is not included in the alignment as it is only a partial sequence.

The YIP1-related ORFs identified in our analysis contain significant stretches of hydrophobic residues. We used the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) to make a prediction of membrane-spanning regions and their orientation for YIP1, YIF1, YGL198W and YGL161C. The TMpred algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins using a combination of several weight matrices for scoring [18]. The results of this analysis are shown in Fig. 2. All of these proteins are small (Yip1p 27.1 kDa, Yif1p 35.5 kDa, Ygl198p 29.1 kDa, and Ygl161p 34.8 kDa) with significant hydrophobic segments which potentially span or are inserted into the membrane. All the Yip1p-related proteins share a predicted topology suggesting that they contain two domains. The N-terminus contains the only significant soluble portion of the protein and constitutes one putative domain. The remainder of the protein constitutes the C-terminal domain and contains several potential membrane-spanning segments. The N-terminal domain is oriented towards the cytosol and the C-terminal domain where the hydrophobic segments are located is largely buried in the membrane. Such a topology has been verified experimentally for Yip1p and Yif1p [9,10,17,19]; the results of our sequence analysis would suggest that this topology is also shared by Ygl198p and Ygl161p.

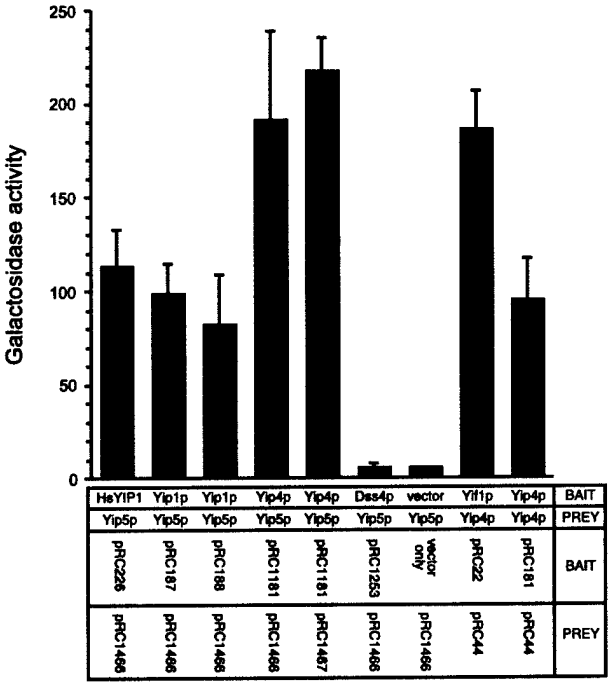


Fig. 5. Yip5p can interact with other YIP1 family members. Pairs of constructs were co-expressed in the reporter strain Y190 and β-galactosidase activity in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The construct pairs are indicated on the *x*-axis; pRC1466 and pRC1467 are two independent prey constructs which express Ygl161p, and pRC44 is a prey construct expressing Yip4p. pRC187 and pRC188 are two independent bait constructs which express Yip1p; pRC226, pRC181, pRC1253 and pRC22 are bait constructs expressing HsYIP1, Yip4p, Dss4p, and Yif1p respectively. Note the slight variability between two independent constructs expressing identical genes, a common feature of this Y2H system.

3.2. *Yip1p family members can interact with Rab proteins*

To investigate the Yip1p-related proteins further, we examined them for potential Rab protein interactions by both Y2H and biochemical pull-down experiments. We constructed a panel of Y2H constructs containing every Rab protein present in *S. cerevisiae* and tested them against the YIP1-related ORFs identified in Fig. 1A. Y2H analysis (Fig. 3A–C) showed that Yif1p, Ygl198p and Ygl161p are capable of interaction with several Rab proteins. In general we found weaker interactions with the Rab proteins Ypt6p and Ypt7p although these constructs still retained the ability to interact with yeast Rab-GDI in this system (data not shown). These data reveal that YIP1-related proteins are capable of binding to determinants shared by many Rab proteins. We have demonstrated this for Yif1p and two novel ORFs, YGL198W and YGL161C. In addition to Rab interactions, our analysis suggests these proteins share a common overall domain topology with a significant hydrophilic N-terminal segment that is cytoplasmically oriented and a largely hydrophobic C-terminal domain (Fig. 2). ORFs named *YIP2* (also termed *YOP1* [10]) and *YIP3* (also termed *PRA1* [20]) are already present in databases, however it is important to note that these ORFs are unrelated in primary sequence to Yip1p. By analogy with Yip1p and to avoid confusion, we suggest that the ORF YGL198W be named Yip4p (Ypt-interacting protein 4) and YGL161C be named Yip5p (Ypt-interacting protein 5).

A common feature of Rab proteins is the prenylation on two C-terminal cysteine residues by the enzyme geranylgeranyl transferase II [5]. To assess the contribution of this post-translational modification to YIP1 family member interaction we generated a Rab construct lacking its C-terminal cysteines. We chose Sec4p as the representative Rab protein as it interacts well with all the YIP1 family members tested. Y2H experiments, shown in Fig. 3D, demonstrated that interaction of Sec4p with Yip5p was completely dependent on its C-terminal cysteines and presumably on correct post-translational modification of the protein. Biochemical experiments (see below) demonstrated that Rab proteins also require prenylation for stable association with Yif1p and Yip4p.

3.3. *Interaction of Yif1p and Ygl198p with Rab proteins in cellular lysates*

To verify the Y2H interactions of YIP1 family members with Rab proteins with an independent technique, we made GST fusions of all yeast Rab proteins. These proteins were expressed under the control of the galactose promoter in yeast, where they would be expected to be correctly post-translationally modified and expressed in cells grown in media with galactose as a carbon source. Expression of a GST fusion of the expected size could be observed for each Rab protein (data not shown). We tested the GST-Rab protein fusions for biochemical interaction by co-precipitation with Yif1p and Yip4p. Yif1p and Yip4p were tagged with an N-terminal MBP fusion and expressed from endogenous promoters. The cellular lysates were incubated with amylose resin for 30 min at 4°C to pull down the MBP-Yif1p or MBP-Yip4p protein. After extensive washing, the bead-bound material was analyzed by SDS–PAGE and Western blotting. The Western blots were probed with anti-GST polyclonal antibody to detect any associated Rab proteins. The results of this analysis are shown in Fig. 4. MBP-Yif1p and Yip4p did not co-precipitate with GST alone, and neither with a Ypt1p construct lacking its C-terminal cysteines which are the sites of prenylation. Both MBP-Yif1p and Yip4p were able to interact with several different Rab proteins. These results parallel the data obtained in the two-hybrid assay and show that Yip1p-related proteins interact with diverse Rab proteins in cellular lysates. Do Rab proteins show different affinities for YIP1 proteins? A precise answer is beyond the scope of this study, however our data (Fig. 4) show that the amount of protein that is precipitated varies between individual Rab proteins. As the expression level of the Rab proteins does not vary significantly this suggests that Rab proteins may have preferences for the YIP1 family member with which they associate. This suggestion must be taken with caution however, as these experiments have utilized tagged proteins which may also influence the observed strength of interaction. If YIP1 family members display differential affinities for each Rab protein this would imply that prenylation, although necessary, is not the sole determinant for interaction.

3.4. *Interactions amongst Yip1p family members*

Yif1p was originally identified as a Yip1p binding partner although its identity as a YIP1-related sequence has not previously been identified [17]. In addition, several Y2H high-throughput screens have identified a plethora of Yip1p-interacting factors amongst which are included YGL198W (*YIP4*) and YGL161C (*YIP5*) [21–23]. These data suggest that Yip1p

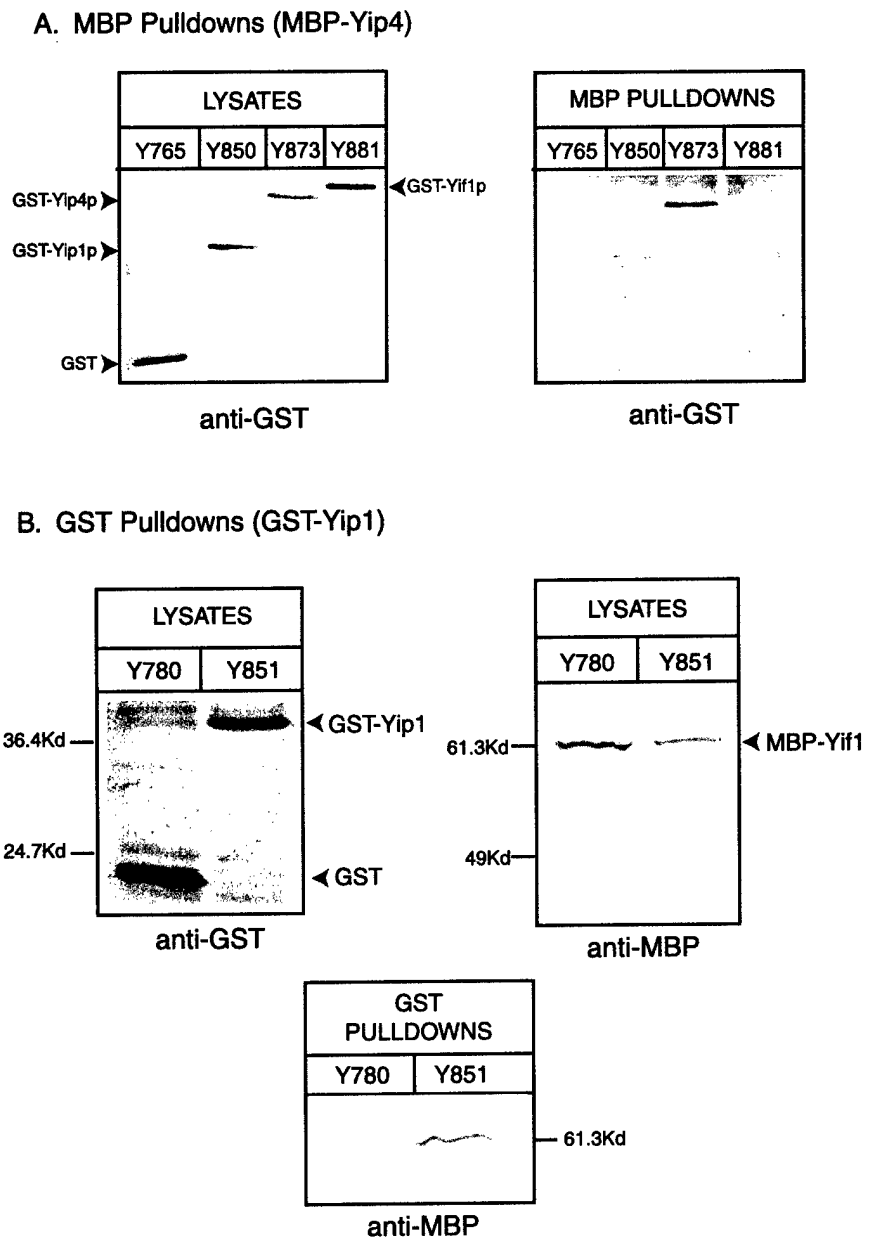
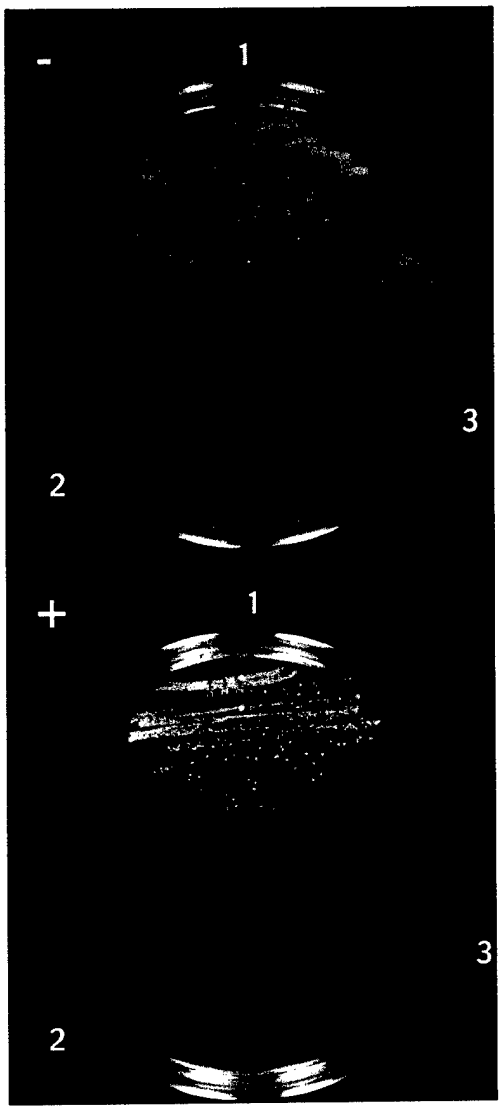


Fig. 6. Biochemical analysis of Yip1p interactions with the Yip1p family members Yif1p and Yip4p. Lysates were prepared from yeast cells expressing (A) GST alone, GST-Yip1p, GST-Yip4p or GST-Yif1p together with MBP-Yip4p (B) GST alone or GST-Yip1p together with MBP-Yif1p (B). Detergent-solubilized total cell lysates were incubated with GST beads (A) or amylose resin (B) for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS–PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST-Yip1p (A) and polyclonal anti-MBP (1:6000) to detect MBP-Yif1p (B). Relevant protein marker sizes are indicated. GST-Yip1p, GST-Yip4p and GST-Yif1p but not GST alone could be detected after MBP-Yip4p pull-downs. MBP-Yif1p could be detected in RCY851 but not RCY780 after glutathione resin pull-downs.

has an ability to physically associate with other YIP1-related sequences. We wished to examine whether YIP1 family members in general share the ability to physically associate amongst themselves. We decided to test these interactions biochemically in deliberate pairwise combinations in both Y2H and biochemical co-precipitation experiments. We chose *YIP5* to test interactions in the Y2H system. The results of this analysis are shown in Fig. 5. Yip5p interacted very strongly with Yip4p and less strongly with Yip1p or the mammalian sequence HsYIP1. Yip4p was also able to self-associate with an interaction level comparable to its interaction with Yip1p. No interactions were observed with an irrelevant plasmid and the Yip5p plasmid showed no autoactivation. As expected, Yip1p and Yif1p also showed strong interactions in the Y2H system.

For the co-precipitation experiments, Yip1p, Yip4p and Yif1p were tagged with GST, Yip4p and Yif1p were tagged with MBP. GST alone was used as a control. An amylose resin pull-down from detergent-solubilized lysates of cells expressing MBP-Yip4p together with either GST alone, GST-



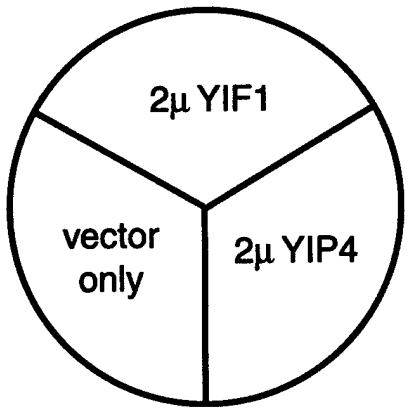


Fig. 7. High-copy plasmids containing the YIP1-related sequence *YIF1* can bypass the requirement for *YIP1*. Cells bearing their only copy of *YIP1* on plasmid containing the counter-selectable marker *URA3* were tested for ability to grow on 5-FOA after transformation with the *YIP1*-related ORFs *YIF1* and *YIP4*. Colonies transformed with multi-copy vectors containing (1) *YIF1*, (2) no insert control, or (3) *YIP4* (YGL198W) were tested for growth on synthetic media with and without 5-FOA to select against retention of the *YIP1* plasmid. Only cells containing multi-copy *YIF1* can survive the loss of the *YIP1*-containing plasmid on 5FOA.

Yip1p, GST-Yip4p or GST-tagged Yif1p revealed that Yip1p, Yif1p and Yip4p could be specifically co-precipitated with Yip4p (Fig. 6A). For Yif1p, we performed the reverse experiment, the GST alone or GST-Yip1p constructs were expressed in cells together with MBP-Yif1p and isolated from detergent solubilized extracts with glutathione agarose. The bead-bound material was probed for associated MBP-Yif1p with an anti-MBP antibody (Fig. 6B). This experiment demonstrated that Yif1p can physically interact with Yip1p, a result which confirms previous findings [17] and demonstrates that the tag used for our experiments does not interfere with protein–protein interactions. Our data confirm and extend the Y2H observations identified in high-throughput screens for Yip1p and suggest that the ability for YIP1 family members to interact amongst themselves is a common feature. Clearly, further experiments are required to ascertain the precise oligomeric nature of these YIP1 family member complexes and determine if the family members have particular preferences for association amongst themselves.

3.5. Overlapping functions of Yip1p family members

Our results demonstrate that YIP1 family members share a common domain topology, bind to Rab proteins in a prenylation-dependent manner and can physically associate amongst themselves. To what extent do the YIP1-related proteins have distinct and overlapping functions? We can begin to answer some of these questions through manipulation of the relevant genes in a genetically tractable organism such as yeast. One of the most stringent tests of function is to ask if one gene can functionally substitute for the deletion of the other. *YIP1* is an essential gene [9] so we tested *YIP4* and *YIF1* for the ability to complement *YIP1* function by asking if these genes could overcome the loss of *YIP1* when expressed from a multicopy plasmid. For this experiment, a strain was generated where the genomic copy of *YIP1* was deleted and viability was maintained by the inclusion of an episomal plasmid containing *YIP1* with a counter-selectable marker, *URA3*. The strain was transformed with a multi-copy plasmid encoding either *YIF1* or *YIP4* and plated on media containing fluoroorotic acid (5-FOA) to select against the *YIP1* gene. Remarkably, *YIF1* overexpression can overcome the loss of *YIP1*; however, *YIP4* was unable to do so (Fig. 7). There are several possible explanations for this result. The overexpression of a gene can suppress defects in other gene products by providing a similar function to that of the absent gene, by providing an alternative pathway or by bypassing the requirement for the absent gene if the suppressor gene lies downstream in the pathway. The fact that *YIF1* can substitute for the absence of *YIP1* indicates that it performs a similar function, further strengthening the suggestion that the YIP1 family may have shared functions and interacting partners. *YIP4* cannot substitute for the loss of *YIP1* indicating that this gene may function upstream of *YIP1* or may act on a different pathway even though these two genes share several potential interacting partners.

Groupings of small membrane proteins with significant hydrophobic segments such as those of the YIP1 family are difficult to establish by conventional means such as BLAST

Table 4
Sequence distances amongst YIP1-related proteins

	1	2	3	4	5	6	
1		30.2	38.3	12.9	14.9	13.3	Yip1p
2	64.3		38.8	12.1	13.2	12.5	HsYIP1
3	59.3	58.2		14.5	13.2	12.8	SpYIP1
4	82.8	83.9	82.5		13.8	11.5	YGL198W
5	84.7	84.5	85.9	79.1		11.9	Yif1p
6	84.2	84.5	85.0	88.2	86.9		YGL161C

The sequence distance table shows the calculated divergence and similarity of each pair of sequences aligned by the Clustal method as outlined in Fig. 1

algorithm searches and must be supported by additional experimental criteria. We propose that for the YIP1-related family, these criteria are: (i) a topology that includes a significant N-terminal hydrophilic domain that faces the cytosol with an hydrophobic C-terminal domain, (ii) the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and (iii) the ability to associate with other members of the YIP1 family. We have demonstrated the unknown ORFs YGL198W (*YIP4*) and YGL161C (*YIP5*) are also Rab-interacting factors and bona fide Yip1p homologs even though they share very little sequence similarity (Table 4). The putative Rab5-interacting protein L1–94 shares two of these criteria [16] and we predict it also to be a member of the YIP1 protein family.

What is the cellular role played by Yip1p-related proteins? One possibility is that they serve as membrane proteins which aid in the recruitment of Rab proteins from the cytosol onto membranes, enabling Rab proteins to be correctly localized and used for many rounds of vesicle transport. Our data suggest that YIP1-related proteins are potential membrane counterparts to Rab-GDI. Similarly to Rab-GDI, they are biochemically capable of interacting with different Rab proteins in a manner dependent on the C-terminal prenylation, perhaps indicating that they can compete with Rab-GDI for Rab protein association. Although there is a plethora of evidence indicating that Rab proteins act downstream of vesicle budding, it is becoming apparent that Rab proteins may also play critical roles in vesicle biogenesis [2]. One rationalization for this may be that a functional vesicle must be equipped with the membrane components required for tasks at a later stage. V-SNAREs, for example, are required for fusion with the acceptor membrane, so these proteins must be included into nascent vesicles with high fidelity. Rab proteins too must be incorporated into the transport vesicle, implying a link between the Rab recruitment machinery and vesicle biogenesis. In support of this idea, Yip1p has been observed to interact with the SNARE protein *TLG1* [23] and we have recently obtained information that Yip1p will interact with the v-SNARE *SNC2* in the Y2H system (unpublished data). Although these data are preliminary and we do not know how far this extends to other YIP1 family members, it is tempting to speculate that there is a functional significance to this interaction. Further strengthening this suggestion is the finding that Yip1p and Yif1p have been observed to be selectively packaged into COPII vesicles in vitro [24], perhaps providing a link between YIP1 family members, Rab proteins and the vesicle biogenesis machinery. Clearly much remains to be understood about these important and intriguing membrane proteins.

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Saccharomyces cerevisiae Pra1p/Yip3p Interacts with Yip1p and Rab Proteins

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The regulation of membrane traffic involves the Rab family of Ras-related GTPases, of which there are a total of 11 members in the yeast *Saccharomyces cerevisiae*. Previous work has identified PRA1 as a dual prenylated Rab GTPase and VAMP2 interacting protein [Martinic *et al.* (1999) *J. Biol. Chem.* 272, 26991–26998]. In this study we demonstrate that the yeast counterpart of PRA1 interacts with Rab proteins and with Yip1p, a membrane protein of unknown function that has been reported to interact specifically with the Rab proteins Ypt1p and Ypt31p. Yeast Pra1p/Yip3p is a factor capable of biochemical interaction with a panel of different Rab proteins and does not show *in vitro* specificity for any particular Rab. The interactions between Pra1p/Yip3p and Rab proteins are dependent on the presence of the Rab protein C-terminal cysteines and require C-terminal prenylation. © 2002 Elsevier Science

Key Words: **PRA1; *YIP3;* GDI; Rab; membrane traffic; yeast; *YIP1*.**

Rab GTPases form the largest branch of small GTPases in the Ras superfamily and are found in all eukaryotic organisms (1). Rab proteins perform essential functions in different membrane transport pathways of the cell such as vesicle biogenesis (2), targeting and fusion of membrane-bound containers (3), and the association of organelles with motor proteins (4).

As with other members of the Ras superfamily, the intrinsic interconversion rates between the GDP- and GTP-bound forms of the molecule are slow, and are regulated by accessory factors such as Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs). In addition to their cycle of nucleotide binding and hydrolysis, Rab proteins also undergo cycles of membrane association and dissociation. Rab proteins stably attach to membranes by virtue of their post-translational prenylation modification; the attachment of two C20 geranylgeranyl groups onto C-terminal cysteines of the protein. The Rab protein can be removed from the membrane through the action of Rab-GDI (GDI). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety (5). The heterodimer of GDP/Rab-GDI enables the Rab protein to be retrieved through the cytosol back onto membranes. The membrane recruitment reaction of Rabs is highly specific, each organelle of the secretory and endocytic pathways is found to associate with a particular Rab protein(s).

To date, many of the Rab accessory factors that have been identified are soluble proteins whose activity can be assigned to defined classes such as effectors, GEFs, GAPs etc. based on their effect on the GDP-GTP interconversion rates. Recently, several Rab-interacting membrane proteins have been identified. These include, Yip1p, PRA1, rab5ip, and Yop1p (6–9). The existence of such proteins raises a question as to their effect on the Rab GTPase cycle of nucleotide binding and localization. There are at least two intervention points in the Rab cycle which may require membrane proteins. The first of these is the dissociation of the cytosolic Rab-GDI heterodimer and subsequent recruitment of the free Rab protein onto membranes. This reaction is specific and is accompanied by the release of GDI, hence the factor that mediates this event has been termed GDI displacement factor (GDF) (10, 11). The second intervention point may be a membrane recycling factor which aids in Rab membrane dissociation. Although GDI is capable of removing Rabs from membranes *in vitro,* this process may be aided *in vivo* by a membrane-associated recycling factor (12).

Rat PRA1 was isolated previously as a Rab3/Rab1 interacting protein (7) however the ability of its yeast homolog Pra1p/Yip3p to physically interact with Rab proteins has not been tested to date. We have tested Pra1p/Yip3p for specificity of the interaction between Yip1p and Rabs. We find that mutations preventing C-terminal prenylation can prevent association of Rabs and Pra1p/Yip3p. Furthermore, we find that the binding of Rab proteins to Pra1p/Yip3p is nonspecific; *in*

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TABLE 1

S. cerevisiae Strains Used in This Study

Strain	Genotype	Source
RCY427	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1-10}$*GST*	This study
RCY442	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT7*	This study
RCY539	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YIP1*	This study
RCY693	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT10*	This study
RCY694	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT11*	This study
RCY695	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT31*	This study
RCY696	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT32*	This study
RCY697	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT52*	This study
RCY698	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT6*	This study
RCY699	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-SEC4*	This study
RCY700	*MATa ura3-52 leu2-3, 112:LEU2* $P_{GAL1/10}$*GST-YPT1ΔC*	This study
RCY701	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT1*	This study
RCY749	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YPT7* [MBP-Pra1p/Yip3p pRS426 pRC1050]	This study
RCY849	*MATa ura3-52 leu2-3, 112::LEU2* $P_{GAL1/10}$*GST-YIP1* [MBP-Pra1p/Yip3p pRS426 pRC1050]	This study
Y190	*MATa gal4Δ gal80Δ trp1-901 ade2-101 ura3-52 leu2-3, 112 URA3::GAL10 → LacZ, LYS2::GAL10 → HIS3 cyh*^{R}	Elledge laboratory

vitro, Pra1p/Yip3p will associate with a variety of Rabs. In addition to Rab proteins, Pra1p/Yip3p also associates with Yip1p and we demonstrate this interaction in cellular lysates.

MATERIALS AND METHODS

Yeast strains and media. The *S. cerevisiae* strains used in these studies are listed in Table 1.

Two-hybrid assay. The ORF sequences were subcloned into pAS1-CYH2 or pAS2-1 and pACTII for "bait" and "prey" constructs respectively. The yeast strain Y190 was used to assay for interacting clones (13). pAS1-CYH2 constructs pRC38, pRC22, pRC27, pRC34, pRC33, pRC804, pRC805, pRC9.8, pRC29, pRC31, pRC25, pRC787, pRC762 express Ypt7p, Yif1p, Ypt11p, Ypt52p, Ypt53p, Ypt1p, Ypt51p, Sec4p, Ypt31p, Ypt32p, Ypt10p, canine Rab1A, and human Rab5, respectively. pACTII constructs pRC40, clone 11.1 express Yip3p exon 2, and Yip1p, respectively.

Coprecipitation experiments. Rab proteins as indicated were expressed as GST-fusion proteins under the control of the $GAL_{1/10}$ promoter in yeast. These strains coexpressed a plasmid containing MBP-tagged Yip3p. The experimental protocol was as described in (9). Strains used for pulldown experiments were grown overnight in 50 ml of selective medium containing galactose as carbon source (SGal) to an absorbance of ~0.7 A_{600}. Cells were harvested by centrifugation at 4°C and washed in 1 ml of ice-cold buffer (10 mM Tris, pH 7.5, 10 mm NaN_3). For all pulldowns, cell pellets were resuspended in 100 μl of ice cold lysis buffer (20 mM KPi, pH 7.5, 80 mM KCl, 1 mM EGTA, 2% glycerol, 0.8% Tween 20) containing protease inhibitors (10 mM PMSF, 10 μg/ml pepstatin A) before lysis with glass beads. A total detergent solubilized extract was generated by incubating lysates with an additional 1 ml of lysis buffer for 10 min at 4°C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microfuge for 5 min at 13,000 rpm. Samples were incubated with rocking for 30 min at 4°C with 20 μl of amylose resin (New England Biolabs). The bead-bound material was washed with four times with lysis buffer. Similar procedures were followed for GST-pulldowns except glutathione *S*-Sepharose resin (Pharmacia) was used to isolate the GST-tagged proteins. Proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were analyzed by 10% SDS–PAGE gel electrophoresis and Western blotting with anti-GST antibody to detect the presence of the GST-tagged Rab proteins (for these purposes the anti-GFP antibody Santa Cruz Cat. No. SC-8334, lot G030 was used, this antibody recognized GST in Western blots with far higher avidity than GFP). -Anti-MBP antibody (gift of G. R. Whittaker) was used at 1:6000 to detect MBP-tagged proteins. Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories) were added in blocking buffer, followed by washing and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

Rab protein expression under the control of the $GAL_{1/10}$ promoter was achieved by subcloning the ORF containing the Rab protein in frame with GST into the vector pRC337. GST–Ypt10p, –Ypt11p, –Ypt31p, –Ypt32p, –Ypt52p, –Ypt6p, –Sec4p, –Ypt1ΔC, –Ypt1p, –Yip1p were expressed from the constructs pRC696, pRC697, pRC698, pRC699, pRC700, pRC701, pRC702, pRC711, pRC1016, and pRC726, respectively. These constructs were linearized with a restriction enzyme and integrated into the genome at the *LEU2* locus. Expression of a GST fusion protein of the correct M_r was determined by growing the cells in media containing 2% galactose as a carbon source. The plasmid containing MBP tagged Yip3p (pRC1050) was constructed by overlap PCR to insert a MBP tag cassette immediately after the initiating methionine in order to express the fusion protein under the control of an endogenous promoter and terminator in the yeast vector pRS426. The PRA1/YIP3 template used for the PCR was an intronless version of the gene created with the primers RNC77 (5′-TTCTATTACCAGAGTACTTGGTATCGAATTGTTTCATTTGAG-3′) and RNC78 (5′-CGATACCAAGTACTCTGGTAATAGAATTTTACAGC-3′) in order to precisely eliminate the intron with no change in coding sequence.

RESULTS AND DISCUSSION

Pra1p/Yip3p Interacts with Multiple Rab Proteins

Martincic *et al.* (7) have previously identified Rat PRA1 as a factor that interacts specifically with Rab3 and Rab1. We wished to extend these observations to the yeast counterpart of PRA1. We performed a deliberate pairwise testing of constructs; including every known Rab ORF (11 total) in *S. cerevisiae.* Interactions

TABLE 2

Pattern of Two-Hybrid Interactions of Rabs with YIP3/PRA1

	Prey		
Bait	*YIP3*	*YIP3* exon 2	*GDI1*
YPT6	+++	+++	++
YPT7	−	−	+
YPT11	+++	+++	+/−
YPT52	+++	+++	+++
YPT53	+++	+++	+++
YPT1	+++	+++	+++
YPT51	+++	+++	+++
SEC4	+++	+++	+++
SEC4ΔC	−	−	−
YPT31	+++	+++	+++
YPT32	+++	+++	+++
YPT10	+++	+++	+++
Rab1	+++	+++	+++
Rab5	+++	+++	+++

Note. β-Galactoside activity was determined by filter assay. Pairs were coexpressed in the receptor strain Y190. Plus represents a positive activity rated according to the following criteria (+++) activity detected after 30 min, (++) activity detected after 90 min, and (+) activity detected after 5 h, and minus (−) is a negative indication of activity. At least 30 independent transformants were tested for each pair.

with Pra1p/Yip3p were observed for all yeast Rabs (Table 2) except for *YPT7* and also the mammalian Rabs, Rab1 and Rab5. These data show that Pra1p/Yip3p interacts with multiple Rab proteins from different species.

The Y2H results were confirmed with an independent method of detecting protein-protein interactions. For these experiments (Fig. 1A) a representative selection of GST-tagged Rab proteins were expressed in cells containing MBP-tagged Yip3p. Untagged GST was expressed as a negative control. The cellular lysates were incubated with amylose resin for 30 min at 0°C to pull-down the MBP–Yip1p protein. After extensive washing, the bead-bound material was analyzed by SDS–PAGE and Western blotting. The Western blots were probed with anti-GST polyclonal antibody to detect any associated Rab proteins. Association of Pra1p/Yip3p was detected with GST–Sec4p, GST–Ypt1p, GST–Ypt6p, GST–Ypt10p, GST–Ypt31p, GST–Ypt32p, and GST–Ypt52p but not to GST alone, GST–Ypt1ΔC or GST–Ypt7p. These results parallel the data obtained in the two-hybrid assay and show that Pra1p/Yip3p in cellular lysates binds to diverse Rab proteins in a manner dependent upon C-terminal prenylation. Ypt7p was the only Rab protein not to interact with Pra1p/Yip3p in cellular lysates. To eliminate the possibility this was due to expression levels of the GST–Ypt7p construct we analyzed the expression levels of this construct in cellular lysates. These data are shown in Fig. 1B, which shows that GST–Ypt7p, GST–Ypt1ΔCp, GST–Ypt1p, and GST alone were expressed at comparable levels in the respective cellular lysates. An equivalent amount of MBP–Yip3p was precipitated in these experiments as revealed by an anti-MBP antibody probe of the membranes. We conclude that Ypt7p is unique amongst Rab proteins in its inability to bind to Pra1p/Yip3p in cellular lysates. Perhaps Ypt7p does not possess the Pra1p/Yip3p binding motif shared by all other Rab proteins. Other possible explanations are that the Pra1p/Yip3p binding site on Ypt7p is masked by association with a Ypt7p-specific factor or that interactions cannot be detected with the tagged constructs used in our system. Purification of Pra1p/Yip3p and demonstration of the direct nature of its interaction with Rab proteins is required for further clarification of this question.

YIP3/PRA1 is unusual amongst yeast genes in that the gene organization consists of two exons, potentially reflecting domain organization of the protein. We used the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) to make a prediction of membrane-spanning regions and their orientation for YIP3. The TMpred algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins using a combination of several weight-matrices for scoring (14). The results of this analysis are shown in Fig. 2. Pra1p/Yip3p is a small 19.4-kDa protein with significant hydrophobic segments that potentially span or are inserted into the membrane. The predicted topology for Pra1p/Yip3p suggests that it exists with a significant soluble N-terminal domain that is oriented toward the cytosol and a C-terminal domain where the hydrophobic segments are located. The topology for yeast Pra1p/Yip3p is very similar to that of mouse PRA1 which has recently reported to be a polytopic membrane protein with four transmembrane segments and a cytosolic N-terminus. Surprisingly, rat PRA1 has been reported to be present in both high speed supernatant and pellet fractions (15). It is difficult to imagine how a polytopic membrane protein can be present in a cytosolic fraction devoid of membranes. Perhaps rat PRA1 exists as a multimeric soluble complex where protein acyl motifs replace the environment of the lipid bilayer enabling the to exist in a cytosolic state? We tested the domain represented by exon 2 in isolation for Rab protein interaction. The exon 2 domain replicated the Y2H interactions observed for full length YIP3/PRA1, suggesting that Rab proteins bind to the C-terminal hydrophobic domain and exclude a role for the N-terminus represented by exon 1 (Table 2).

Our data demonstrate that Pra1p/Yip3p interacts with multiple different Rab proteins. This data, together the fact that the human homolog PRA1 interacts with the v-SNARE protein synaptobrevin, builds up a picture of Pra1p/Yip3p playing a role in membrane traffic events. In addition to Rab proteins, Pra1p/

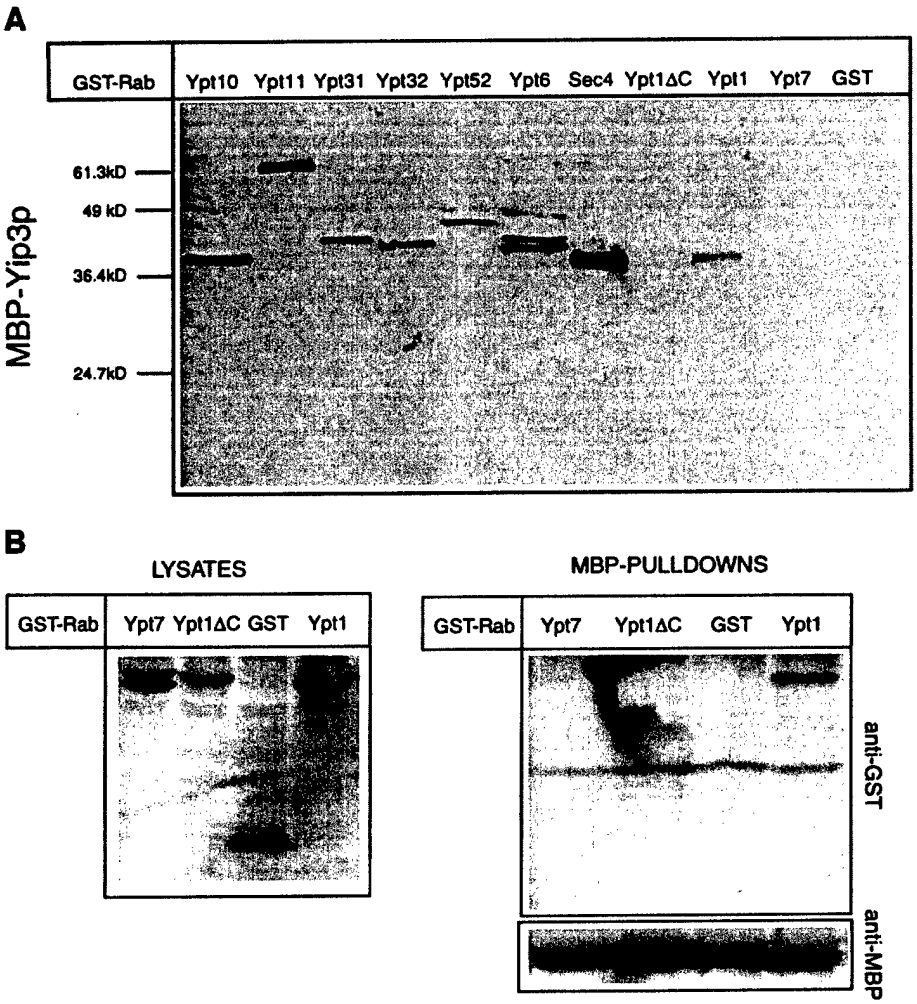

FIG. 1. Biochemical analysis of Pra1p/Yip3p interactions with Rabs: MBP–Yip3p interacts with fully post-translationally modified Rab proteins. (A) Lysates were prepared from cells expressing various GST–Rab constructs as indicated. Detergent-solubilized lysates were incubated with amylose resin for 30 min at 4°C as described under Materials and Methods to pull-down the MBP–Yip3p. After four washes, the bead-bound material was subject to SDS–PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST–Rab proteins. Relevant protein marker sizes are indicated. (B) MBP–Yip3p fusion protein was purified from lysates prepared from cells expressing GST–Ypt7p, GST–Ypt1ΔCp, GST alone, or GST–Ypt1p. Both total cell lysates and the MBP pulldowns were Western blotted with anti-GST antibodies to detect the relative abundance of the GST fusion proteins. The MBP-pulldowns were additionally subject to Western blotting with anti-MBP antibodies to confirm the precipitation of MBP–Yip3p on the amylose resin. Only GST–Ypt1p, but not GST–Ypt7p, GST–Ypt1ΔCp, or GST were observed to coprecipitate with MBP–Yip3p although these constructs were expressed at similar levels in the cellular lysates.

Yip3p has also been shown to interact with Rho and Ras small GTPases in a manner dependent on C-terminal prenylation (16). The *in vivo* significance of this data is unclear. Although conserved in evolution and ubiquitously expressed (17), *PRA1/YIP3* is not an essential gene in yeast perhaps indicating its function is in a supporting or mediator role. By binding prenylation groups on small GTPases, or other prenylated molecules, Pra1p/Yip3p has been suggested to act as a carrier protein mediating the intracellular movement of prenylated proteins (16), a function it could carry out alone or in concert with other binding partner(s). One possible hypothesis for Yip1p function is suggested by the features of Pra1p/Yip3p interaction with Rabs demonstrated in this study, namely that interactions are (i) nonspecific and (ii) require the C-terminal cysteines which are the recipient sites for double geranylgeranylation. These features exactly mirror the requirements for Rab interaction with Rab-GDI and suggest that Pra1p/Yip3p can directly compete with Rab-GDI for Rab protein interactions on the membrane. Such an outcome has been suggested by the study of Hutt *et al.* (15) although the physiological significance of this data is unclear. In addition, the finding that Pra1p/Yip3p can bind prenylated Rho small GTPases suggest that Pra1p/Yip3p could simi-

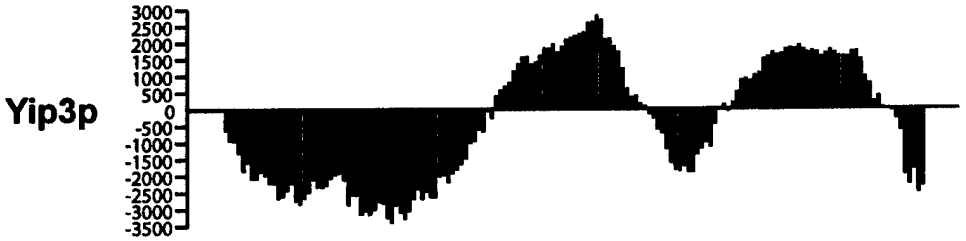

FIG. 2. TMpred plot of Pra1p/Yip3p. The TMpred plot of Pra1p/Yip3p was generated using the program TMpred with a 17-residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix and shows the relative location of the *y*-axis the relative hydrophobicity (positive values) or hydrophilicity (negative values). Sequence data indicate a predicted topology for Pra1p/Yip3p with a significant soluble N-terminal domain that is oriented toward the cytosol and a C-terminal domain where the hydrophobic segments are located.

larly directly compete with Rho-GDI for Rab protein interactions on the membrane.

Our biochemical data reveal that the yeast PRA1-related protein is capable of binding to a common determinant shared by multiple Rab proteins with the exception of Ypt7p. These data are in agreement with the finding that rat PRA1 interacts specifically with Rab3A and Rab1. Rat PRA1 does not interact with Rho or Rac although is able to bind Rab proteins where the usual di-cysteine motif has been replaced with the CAAX motif for mono-geranylgeranylation (7). This is in contrast to the finding that yeast Pra1p/Yip3p interacts with Rho proteins (16), although this study did not examine or compare the interaction with Rab proteins. Our results demonstrate these Pra1p/Yip3p interactions are conserved across evolution, not only does YIP3 interact with yeast Rab proteins, it will similarly interact with a mammalian Rab protein and similar interactions by Y2H have been reported for a human homolog of PRA1 (18). Demonstration of the direct or indirect nature of the PRA1 interactions is required for further resolution of these questions since all experiments carried out to date have been performed in cell extracts.

Pra1p/Yip3p Interacts with Yip1p

In addition to Rab proteins, Pra1p/Yip3p has also been observed to interact with Yip1p by Y2H (19–21). We decided to test this interaction biochemically in co-precipitation experiments. For these experiments, Yip1p was tagged with GST and Yip3p was tagged with MBP. GST alone was used as a control. An amylose resin pull-down from detergent solubilized lysates of cells expressing either MBP–Yip3p together with GST alone or GST-tagged Yip1p revealed that Yip1p could be specifically co-precipitated (Fig. 3). Our data therefore confirm and extend the Y2H observations identified in high throughput screens for Pra1p/Yip3p.

In addition to pleiotropic Rab interactions *in vitro*, our analysis suggests that YIP3 is able to interact with the essential membrane protein Yip1p. Human PRA1 can also interact with Epstein–Barr virus BHRF1, a homologue of Bcl-2 (22) and Piccolo, a novel component of the presynaptic cytoskeletal matrix (23). Yip1p has also been observed by biochemical experiments and Y2H to interact with both Yif1p and Yop1p (9, 24); and by Y2H with YIP3, YGL198W, YGL161C, YPL095C, GCS1, and YLR324W (19–21). The relevance of these demonstrated and potential interactions is obscure, although Yip1p, Yif1p and Pra1p/Yip3p have been observed to be selectively packaged into COPII vesicles *in vitro* (25), perhaps providing a link between YIP1 family members, Rab proteins and the vesicle docking and fusion machinery. Further work will be required to clarify the complex and confusing issues surrounding these conserved proteins and to understand the physiological role of PRA1/YIP3 and its mechanism of action.

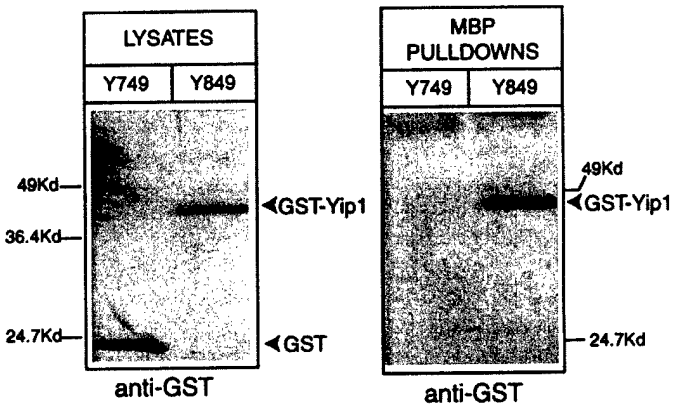

FIG. 3. Biochemical analysis of Pra1p/Yip3p interactions with Yip1p. Lysates were prepared from cells expressing either GST alone or GST–Yip1p together with MBP–Yip3p. Detergent solubilized lysates were incubated with amylose resin for 30 min at 4°C as described under Materials and Methods. After washing, the bead-bound material was subject to SDS–PAGE and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST–Yip1p. Relevant protein marker sizes are indicated. GST–Yip1p was detected in RCY849 but not on RCY749 after MBP-pulldowns.

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Dual Prenylation Is Required for Rab Protein Localization and Function

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The majority of Rab proteins are posttranslationally modified with two geranylgeranyl lipid moieties that enable their stable association with membranes. In this study, we present evidence to demonstrate that there is a specific lipid requirement for Rab protein localization and function. Substitution of different prenyl anchors on Rab GTPases does not lead to correct function. In the case of *YPT1* and *SEC4*, two essential Rab genes in *Saccharomyces cerevisiae*, alternative lipid tails cannot support life when present as the sole source of *YPT1* and *SEC4*. Furthermore, our data suggest that double geranyl-geranyl groups are required for Rab proteins to correctly localize to their characteristic organelle membrane. We have identified a factor, Yip1p that specifically binds the di-geranylgeranylated Rab and does not interact with mono-prenylated Rab proteins. This is the first demonstration that the double prenylation modification of Rab proteins is an important feature in the function of this small GTPase family and adds specific prenylation to the already known determinants of Rab localization.

INTRODUCTION

Rab proteins are GTPase superfamily members that regulate membrane trafficking through the secretory and endocytic pathways. The Rab proteins represent the numerically largest subgroup of the Ras superfamily, and it is thought that each stage of membrane transport through both constitutive cellular pathways and in differentiated cells with specialized organelles is associated with one or more Rab proteins (Pfeffer, 2001). The mechanism by which Rab proteins act to regulate membrane-trafficking events is not fully understood. A plethora of different effector proteins have been identified connecting the activated Rab proteins to a variety of cellular events such as cytoskeletal dynamics, phosphatidylinositol signaling events, protein kinase-mediated signal transduction, and the establishment of cell cycle-linked spatial cues (Ren *et al.*, 1996; Finger *et al.*, 1998; Christoforidis *et al.*, 1999; Nielsen *et al.*, 1999). Given the complexity of the exocytic and endocytic trafficking pathways, it makes sense for cells to link Rab protein activation to a variety of different outcomes depending on the requirements of the various organelles involved.

Recently, a consensus seems to be emerging that one commonality of Rab protein function is to participate in the tethering of a vesicle or membrane transport carrier (Pfeffer, 1999). Tethering refers to the process by which the membrane bound transport carriers dock onto the acceptor compartment. Tethering is the prelude to, and initiator of the cascade of events that terminate in a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-mediated membrane fusion event. Even tethering may be controlled by Rab proteins through diverse mechanisms; for example, the tethering of constitutive post-Golgi vesicles at the plasma membrane is a transient event and very different from the rapid, signal-mediated fusion of synaptic post-Golgi vesicles that may exist in the tethered state for a prolonged time period (Wang *et al.*, 1997). These examples, in turn, differ in their requirements from the fusion of post-Golgi vesicles with the plasma membrane of budding yeast, where tethering events must be spatially regulated and are coordinated with cell cycle progression.

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Abbreviations used: 5-FOA, 5-fluorooroic acid; FTase, farnesyl transferase; GDI, GDP-dissociation inhibitor; GGTase I, geranylgeranyl transferase type I; GGTaseII, type II geranylgeranyl transferase; GFP, green fluorescent protein; mAb, monoclonal antibody; PCR, polymerase chain reaction; REP, Rab escort protein; Y2H, yeast two-hybrid.

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
BY85	MATa/α *ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 his3Δ200/his3Δ200 YPT1/YPT1ΔHIS3*	Brennwald laboratory
BY86	*MATa/α ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 his3Δ200/his3Δ200 SEC4/SEC4ΔHIS3*	Brennwald laboratory
AG6	*LEU2::SEC4$^{(L7,HV)YP}$ SEC4Δ::HIS3 his3Δ200 ura3-52*	Brennwald laboratory
BY24	*MATa/α* ura3-52/ura3-52 leu2-3, 112/leu2-3, 112	Brennwald laboratory
NY605	*MATa ura3-52 leu2-3, 112*	Novick laboratory
RCY1507	*MATα ura3-52 leu2-3, 112 his3Δ200 SEC4ΔHIS3* [YCP50 *SEC4*]	This study
RCY1510	*ura3-52 leu2-3, 112 his3Δ200 YPT1ΔHIS3* [pRS316 (pRC1762) *YPT1*]	This study
RCY 1530	*MATa/α ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 YIP1/YIP1ΔKAN^R* [YCP50 (pRC1245) YIP1]	This study
RCY1610	*MATa ura3-52 leu2-3, 112 YIP1ΔKAN^R* [YCP50 (pRC1245) YIP1]	This study
RCY1764	*MATa ura3-52 leu2-3, 112 YIP1ΔKAN^R* [pRS315 *yip1-4*]	This study
RCY1760	*MATa ura3-52 leu2-3, 112 YIP1ΔKAN^R* [pRS315 *YIP1*]	This study
Y190	*MATa gal4Δ gal80Δ trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL10 → LacZ, LYS2::GAL10 → HIS3 cyh^R*	Elledge laboratory

Ras GTPases function as regulatory switches where the GDP-bound is the ground or "off" state and GTP-bound is the activated state (Vetter and Wittinghofer, 2001). It is still unclear whether Rab proteins function as binary switches in a similar manner to Ras with a single GTP turnover event for each round of transport. An alternative modality is suggested by analogy to the Rho family GTPase CDC42 where it is the rate of cycling, rather than the lifetime of the activated state, that is important for initiation of downstream events (Rybin *et al.*, 1996; Lin *et al.*, 1997).

One characteristic that Rab proteins do share with other members of the Ras superfamily is that these proteins are posttranslationally modified by the covalent attachment of isoprenoids on cysteine residues at the C terminus (Seabra, 1998). For the Ras, Rho, and Rab families, there are three major types of isoprenylation reactions mediated by three prenyl transferases that are conserved from yeast to human (for review, see Liang *et al.*, 2002). The cysteine-containing motifs at the C terminus dictate the type of isoprenylation received by the small GTPase. A CAAX box where C is cysteine, A is aliphatic residue, and X is A, C, E, M, S, or V such as in H-Ras, K-Ras, and yeast Ras1p and Ras2p, is modified by farnesylation (C15 isoprenoid) by farensyl transferase (FTase). When X is leucine or a hydrophobic residue, typically found in Rho proteins such as CDC42, this is as substrate for geranylgeranyl transferase I (GGTase I), which attaches a C20 isoprenoid moiety. Rab proteins fall into a special category. The majority of them contain two cysteine residues at the C terminus in one of the following sequences: CXC, CC, CCX, CCXX, or CCXXX. The cysteine residues are subject to isoprenylation with two geranylgeranyl moieties catalyzed by geranylgeranyl transferase II (GGTase II). All the prenyltansferase enzymes consist of two subunits, however, in the case of GGTaseII, there is a third subunit, Rab escort protein (REP), which does not participate in the catalytic reaction but serves as a chaperone to introduce the prenyltransferase to its Rab protein substrate (Desnoyoyers *et al.*, 1996).

Ras, Rho, and Rab superfamily members can be found in both membrane-associated and cytosolic pools. It is clear that prenylation is a necessary modification for the protein to be present in the membrane-bound pool, Ras superfamily members with mutations in their C-terminal cysteines that cannot be prenylated are soluble and nonfunctional (Walworth *et al.*, 1989). Such experiments have propagated the view that the sole function of prenylation is to confer hydrophobic character onto a cytosolic protein, giving the recipient protein the physical ability to make a stable attachment with a lipid bilayer. In this study, we have focused on the question of what role, if any, is played by the particular type of lipid modification. Using *Saccharomyces cerevisiae* as a model system, we have examined the effect of different lipid modifications on Rab protein localization and function.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. cerevisiae* strains used in these studies are listed in Table 1. All yeast strains were manipulated as described by Guthrie and Fink (2002). Yeast expressing various green fluorescent protein (GFP)-Rab proteins (both wild-type and prenylation mutants) were created by transforming the appropriate plasmids (Table 1) into NY605. Yeast strains were streaked on selection media plates and incubated at 30°C. Liquid media cultures were grown at room temperature. A single colony from each strain was inoculated into 5 ml of selective medium and grown to stationary phase. For fluorescence microscopy, selective media were inoculated with an aliquot of the stationary culture and grown to early to mid-log phase. Cells were then incubated for 5 min with 5 μg/ml Hoechst for nuclear visualization before image capture. For Triton X-114 partition experiments, an aliquot of the stationary phase was inoculated into 50 ml of selective media, and the cells were grown to mid-log phase. Turbidity measurements were made using a Thermo Spectronic Genesys (Rochester, NY) 10UV spectrophotometer at 600 nm.

Plasmid Constructs

Plasmid constructs and oligonucleotides are listed in Tables 2 and 3. Yeast Rab genes were cloned under the control of an endogenous promoter and terminator with yeast-enhanced GFP (GenBank accession no. U73901) fused in frame at the N terminus by polymerase chain reaction (PCR) and cloned into the CEN *LEU2* vector pRS315 or pRS316 to generate plasmids containing GFP-tagged genes. Plasmids with wild-type Rab genes were used as templates to generate

Table 2. Plasmids used in this study

Plasmid name	Construct	Source
pRC651	GFP-SEC4 pRS315 CEN *LEU2*	This study
pRC2098	GFP-SEC4 pRS316 CEN *URA3*	This study
pRC1822	GFP-SEC4^{CTIM} pRS315 CEN *LEU2*	This study
pRC1268	GFP-SEC4^{CTIM} pRS316 CEN *URA3*	This study
pRC1286	SEC4^{CTIM} pRS426 2μ *URA3*	This study
pRC1842	GFP-SEC4^{CIIL} pRS315 CEN *LEU2*	This study
pRC1552	GFP-SEC4^{CIIL} pRS316 CEN *URA3*	This study
pRC1860	GFP-SEC4^{C214S} pRS315 CEN *LEU2*	This study
pRC1861	GFP-SEC4^{C214S} pRS316 CEN *URA3*	This study
pRC1862	GFP-SEC4ΔCC pRS315 CEN *LEU2*	This study
pRC1863	GFP-SEC4ΔCC pRS316 CEN *URA3*	This study
pRC1820	*SEC4* pRS315 CEN *LEU2*	This study
pNB139	*SEC4* YCP50 CEN *URA3*	Novick lab
pRC1292	SEC4^{CTIM} pRS316 CEN *URA3*	This study
pRC1824	SEC4^{CTIM} pRS315 CEN *LEU2*	This study
pRC1743	SEC4^{CIIL} pRS315 CEN *LEU2*	This study
pRC1728	SEC4^{CIIL} pRS316 CEN *URA3*	This study
pRC1856	SEC4^{C214S} pRS315 CEN *LEU2*	This study
pRC1857	SEC4^{C214S} pRS316 CEN *URA3*	This study
pRC1858	SEC4ΔCC pRS315 CEN *LEU2*	This study
pRC1859	SEC4ΔCC pRS316 CEN *URA3*	This study
pRC2100B	GFP-YPT1 pRS315 CEN *LEU2*	This study
pRC1840	GFP-YPT1^{CTIM} pRS315 CEN *LEU2*	This study
pRC1752	GFP-YPT1^{CIIL} pRS315 CEN *LEU2*	This study
pRC1840A	GFP-YPT1^{CTIM} pRS316 CEN *URA3*	This study
pRC1735	*YPT1* pRS315 CEN *LEU2*	This study
pRC1762	*YPT1* pRS316 CEN *URA3*	This study
pRC1829A	YPT1^{CTIM} pRS315 CEN *LEU2*	This study
pRC1828	YPT1^{CTIM} pRS316 CEN *URA3*	This study
pRC1730	YPT1^{CIIL} pRS315 CEN *LEU2*	This study
pRC1888A	GFP-*YPT1*^{C205S} pRS315 CEN *LEU2*	This study
pRC1889A	*YPT1*^{C205S} pRS315 CEN *LEU2*	This study
pRC1887A	GFP-YPT1ΔCC pRS315 CEN *LEU2*	This study
pRC1884A	YPT1ΔCC pRS315 CEN *LEU2*	This study
pRC1243	GFP-YPT7 pRS316 CEN *URA3*	This study
pRC1272	GFP-YPT7^{CTIM} pRS316 CEN *URA3*	This study
pRC1560	GFP-YPT7^{CIIL} pRS316 CEN *URA3*	This study
pRC650	GFP-YPT6 pRS315 CEN *LEU2*	This study
pRC1556	GFP-YPT6^{CIIL} pRS316 CEN *URA3*	This study
pRC1544	GFP-YPT6^{CTIM} pRS316 CEN *URA3*	This study
pRC680	GFP-VPS21 pRS306 INT *URA3*	This study
pRC1541	GFP-VPS21^{CTIM} pRS316 CEN *URA3*	This study
pRC1964	GFP-VPS21^{CIIL} pRS315 CEN *LEU2*	This study
pRC1462	SEC4^{CTIM} pAS2-1	This study
pRC1798	SEC4^{CIIL} pAS2-1	This study
pRC575	YPT1 pACT2	This study
pRC579	SEC4 pACT2	This study
pRC188	YIP1 pAS1-CYH2	This study
pRC2170	P_{YIP1} → HsYIP1A pRS315 CEN *LEU2*	This study
pRC1992	*yip1*^{E70K} pRS315 CEN *LEU2*	This study
pRC1838b	*YIP1* pRS315 CEN *LEU2*	This study
pRC219	pACT2 HsYIP1A	This study
pRC1803	Rab8 pAS2-1	This study
pRC1801	Rab13 pAS2-1	This study
pRC1802	Rab13 pAS2-1	This study
pRC787	Rab1a pAS2-1	This study
pRC763	Rab5a pAS2-1	This study
pRC2240	Sec7p-T4DsRed pRS316 CEN *URA3*	This study

the GFP-tagged C-terminal prenylation variants. The prenylation mutants with a terminal CTIM sequence were cloned into pRS315 or pRS316 by overlap PCR by placing the C terminus of *RHO3* containing the sequence CTIM and terminator in place of the two terminal cysteines by using the forward primer MC25 and reverse primers with overlap sequence to MC25 for *SEC4* (MC27), *YPT7* (MC26), *VPS21* (MC31), *YPT1* (MC32), and *YPT6* (MC30). The Rab prenylation variants with a terminal CIIL sequence were cloned into pRS315 or pRS316 by overlap PCR by using specific forward and reverse primers with nucleotide sequence coding for CIIL and the endogenous terminators of each Rab. Primers MC54 and MC55 were used for cloning *SEC4*, MC58, and MC59 for *YPT7*, MC60 and MC61 for *YPT6*, and MC64 and MC65 for *YPT1*. For *SEC4*^{C214S}, *YPT1*^{C205S}, *SEC4*$^{\Delta CC}$, and *YPT1*$^{\Delta CC}$, a similar approach was used with primers MC66, MC67, MC68, MC69, MC70, MC71, MC72, and MC73. *VPS21*^{CIIL} was cloned with primer MC75, which anneals to the C terminus of *VPS21* and overlaps with MC74, a primer-encoding CIIL sequence and the terminator of *SEC4*. Untagged wild-type *SEC4* and *YPT1* were constructed with genomic PCR by using primers YFSEC4, YRSEC4, YFYPT1, and YRYPT1 and cloned into pRS315 or pRS316. Untagged prenylation variants of *SEC4* and *YPT1* were cloned into pRS315 and pRS316 by overlap PCR by using YFSEC4 and YFYPT1 and primers as described above for the GFP-tagged variants. The primers RNC200 and RNC201 were used with genomic DNA template to clone full-length GDP-dissociation inhibitor 1 (*GDI1*) into the vectors YEP24 and YCP50. *SEC4*, *SEC4*^{CTIM}, *SEC4*^{CIIL}, *SEC4*^{C214S}, and *SEC4*$^{\Delta CC}$ were cloned into pAS2–1 to create two-hybrid "bait" plasmids. *SEC4*^{CTIM} and *SEC4*^{C214S} were cloned using primers NS1 with YRRHO3, and NS2 with RNC264, respectively, and subcloned in-frame into pAS2-1 vector. SEC4^{CIIL} was cloned by genomic PCR with primers MC56 and MC57 and subcloned into pAS2–1. Other Y2H constructs used have been described previously (Calero *et al.*, 2002). Sec7p was tagged with *Discosoma* red fluorescent protein (DsRed)T4 (Bevis and Glick, 2002) at the C terminus with the linker sequence GGPGG and subcloned into pRS316 with the endogenous promoter and 572 bp from the *ADH1* 3′ region to create pRC2240. A human open reading frame (ORF) encoding a protein with homology to Yip1p was reconstructed by alignment of accession numbers AA171435, AA373289, H83008, N73033, R88629, T71419, and W17013. The ORF was cloned by coupled reverse transcription-PCR by using the reverse transcription primer 5′-GGCCACGCGTCGACTAGTAC(T)$_{17}$ and gene-specific PCR primers 5′-CTGGATCCTCGCAATGTCAGGCTTT-GAAAACTTAAACACGG and 5′-GATGCGCGTCTCGAGTCAAA-AGACGGAAATCAGGGCAAAGAC. Then 250 ng of human skeletal muscle poly(A)$^+$ RNA (BD Biosciences Clontech, Palo Alto, CA) was reverse transcribed with Superscript II according to the manufacturer's protocol. Purified cDNA was used as a template in PCR reactions to amplify human YIP1. The sequence of the human ORF is identical to the previously reported YIP1A (Tang *et al.*, 2001). Oligonucleotides used in this study were from by Integrated DNA Technologies and Sigma Genosys (The Woodlands, TX). DNA sequencing was performed by the Cornell Biotechnology Facility by using dye terminator chemistry on an ABI 373 sequencer (Applied Biosystems, Foster City, CA).

Creation of yip1-4 Thermosensitive Allele

YIP1 gene deletion was carried out using the KAN^R module (Wach *et al.*, 1994) as a selectable marker and the oligonucleotides S1YIP1 and S2YIP1 to precisely eliminate the *YIP1* ORF in the diploid yeast strain BY24. The mutant allele of *YIP1* was generated by a standard plasmid shuffling procedure (Sikorski *et al.*, 1991). Briefly, RCY1610 was transformed with plasmid pRS315 containing the mutant *yip1-4* gene created with primers KAH7 and KAH8 and genomic DNA template. Transformants were selected on synthetic media lacking leucine followed by colony purification on fluoroorotic acid (5-FOA)-containing media. 5-FOA–resistant colonies were tested for temperature-sensitive growth on rich media.

Table 3. Oligonucleotides used in this study

Primer name	Sequence 5′ to 3′
MC25	TGTACCATTATGTAATATAATAAG
MC26	CTTATTATATTACATAATGGTACAAGAATTATTTTCTCCATCTAG
MC27	CTTATTATATTACATAATGGTACAATTTGATTTAGAACTGTT
MC30	CTTATTATATTACATAATGGTACAAGCGCTTTGTTCCTGCTC
MC31	CTTATTATATTACATAATGGTACAAGCACTGTTTGCGCTGGT
MC32	CTTATTATATTACATAATGGTACAGCCCCCACCGGTGTTGGTTAA
YRRH03	TACCGGGCCCCCCCCTCGAGGTCGACGCGTAAATCGTAACCATAGTAAG
YFSEC4	TACCGGGCCCCCCCCTCGAGGTCGACTTAGAACGAAATAAAAGTGCT
MC54	AAATCAAATTGTATTATTTTGTGAAGAAAAGAAGATTTTTGCTTC
MC55	TTCTTCACAAAATAATACAATTTGATTTAGAACTGTTTCC
MC56	CATGCCATGGCATCAGGCTTGAGAACTGTTTC
MC57	ATACTCGAGGCTTCTTTTCTTCACAAAATAA
MC58	AATAATTCTTGTATTATTTTGTGAGCTGTACTACGTCGACCTCGA
MC59	CAAAATAATACAAGAATTATTTTCTCCATCTAGGCGAATATT
MC60	CAAAATAATACAAGAATTATTTTCTCCATCTAGGCGAATATT
MC61	CAAAATAATACAAGCGCTTTGTTCCTGCTCCTCTGCTGTAGA
MC74	TCTTCTTTTCTTCACAAAATAATGCATGCACTGTTTGCGCTGGT
MC75	CATTATTTTGTGAAGAAAAGAAGATTTTTGC
YRSEC4	ACCGGGCCCCCCCCTCGAGGTCGACAAGTAGTTGAATAGTGGATTC
MC70	GGTGGGGGCTGACTGCAGGCCTCTACCTTGCAGACCCATATAATA
MC71	GAGGCCTGCAGTCAGCCCCCACCGGTGTTGGTTAA
MC72	GGGGGCTCTTGTTGACTCGAGGCCTCTACCTTGCAGACCCATATA
MC73	GGCCTCGAGTCAACAAGAGCCCCCGCCCCCACCGGTGTTGGTTAA
YFYPT1	CGGCCGCTCTAGAACTAGTGGATCCTATATTACTTTGTGGAGATTT
YRYPT1	TACCGGGCCCCCCCCTCGAGGTCGACTAAACAAGAGAGATTGGGAAGGAA
MC66	ATTGCTCTTGACTCGAGGTGAACTGGAATTA
MC67	GTTCACCTCGAGTCAAGAGCAATTTGATTTAGAACTGTTTCCG
MC68	ATTCAAATTGACTGCAGGTGAACTGGAATTAC
MC69	CAGTTCACCTGCAGTCAATTTGATTTAGAACTGTTTCCGCT
MC64	GCTTGTTCACAAAATAATACAGCCCCCACCGGTGTTGGTTAA
MC65	GGTGGGGGCTGTATTATTTTGTGAACAAGCGCGCCTCTAC
NS1	ATGGATCCTGTCAGGCTTGAGAACTGTTTCTG
NS2	TTATCTCGAGTCAACAGCAATTTGATTTAGAACTG
RNC262	TAGGATCCGATTGATATTCTTTTTGTTATTCGGAC
RNC263	ATATACTCGAGTTCAGACAAAAATTACCATGAGG
RNC264	ATACTCGAGTCAAGAGCAATTTGATTTAGAACTGTTTCC
RNC200	TTGGAGCCACTATCGACTACGCGATCATGGCGACCAGGCCGTGGGAAGCTTC
RNC201	TGATGCCGGCCACGATGCGTCCGGCGTAAGACTGACAGTTATACCCAAG
YFY1P1	CGGCCGCTCTAGAACTAGTGGATCCCGTATCTCGTTAGTACTTGTT
YRYIP1	TCACACAGGAAACAGCTATGACCATGAAGCTTGACCTTAGAGTACAGACGATG
KAH7	ATAAACCTCCATTACTCGAGGAAATTGGAATAAATTTCG
KAH8	CCTCGAGTAATGGAGGTTTATGTGGATATCCCTTAGTTGAAAGAG
RNC222	TACCGGGCCCCCCCCTCGAGGTCGACAGAATTCGGCATGCCGGTAGAGGTGTGGTC
CC16	CTCGAGAGGGACTAATAGTTGT
CC22	AACTATTAGTCCCTCTCGAGATGTCAGGCTTTGAAAACTTAAACA
MC1	CGGGATCCCATCCAGCATGAATCCCGAA
MC2	CCGCTCGAGGTTTAGCAGCAACCTCCACC
MC3	ATGGATCCCAGCTAGTCGAGGCGCAACA
MC4	ATACTCGAGGTTTAGTTACTACAACACTGATTCCT
RNC256	ATGAATTCGCCAAAGCCTACGACCA
RNC257	ATGGATCCTCAGCCCAGGGAGCAC
RNC258	ATGAATTCGCGAAGACCTACGATTACCTG
RNC259	ATGGATCCTCACAGAAGAACACATCGGAAAAAG
RNC263	CAGAAACAACTCTGGCGCATC
RNC228	CTATGGAACTGCCTCGGTGA
S1YIP1	GCTACAAATTGGACGGGAAGTACTGCAAGACAACTATTAGTCCCTCTCGAGCGTACGCTGCAGGTCGAC
S2YIP1	GTTCAGAAAAACATATATACAAATATCGCCCCTAAGCCAATTCCCTTCAATCGATGAATTCGAGCTCG

Triton X-114 Partition Experiments

Triton X-114 (Roche Diagnostics, Indianapolis, IN) was purified by precondensation as described previously (Bordier, 1981). Then 5 OD units of yeast strains were harvested and washed in 1 ml of TAZ buffer. Postnuclear supernatants were generated by two sequential centrifugation steps for 5 min at 500 × *g*. Then 500 μl of phosphate-buffered saline (PBS) containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1

mM benzamidine, and 10 μg pepstatin A) was added to the post-nuclear supernatants. The samples were incubated for 20 min at 4°C to solubilize membrane proteins. To separate the detergent-enriched and the -soluble phases, samples were incubated for 3 min at 30°C followed by low-speed centrifugation at room temperature. This cycle was repeated two times with the detergent-enriched and -soluble phases individually. The detergent phase was washed twice with PBS containing 0.05% Triton X-114 and the soluble phase with 2% Triton X-114. Samples were then incubated with an equal volume of 10% trichloroacetic acid on ice for 30 min followed by a centrifugation at 4°C for 15 min. The protein pellets were washed twice with 300 μl of cold acetone and resuspended in 15 μl of SDS sample buffer. The samples were then analyzed by SDS-PAGE and Western blot. Affinity-purified α-GFP polyclonal antibody (gift from Pam Silver, Harvard University, Cambridge, MA; Seedorf *et al.*, 1999) and alkaline phosphatase-conjugated anti-rabbit secondary antibody (Bio-Rad, Hercules, CA) were used to detect the GFP-tagged Rab protein. Snc1/2p, an integral membrane protein, was detected with anti-Snc1/2p antisera (gift from P. Brennwald, Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC).

Microscopy

For direct fluorescence microscopy, yeast strains were grown to mid-log phase in selection media. For visualization of the nuclei, the samples were incubated with 5 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR) for 5 min. For immunofluorescence microscopy, cells were grown to early log phase in YPD or selection media. A 2× fixative (2× PBS, 4% glucose, 40 mM EGTA, and 7.4% formaldehyde) was added to an equal volume of medium containing 3 OD units of cells and incubated for 20 min at room temperature. Cells were then collected by centrifugation, resuspended in 5 ml of 1× fixative, and incubated for a further 1 h. The cells were washed twice in 2 ml of spheroplasting buffer (100 mM KPi pH 7.5 and 1.2 M sorbitol) and then incubated in spheroplasting buffer containing 0.2% 2-mercaptoethanol and 0.08 mg/ml zymolyase for 30 min at 37°C with gentle mixing. Then 20 μl of the cell suspension was placed on individual wells of a polylysine-coated printed microscope slides (Carlson Scientific, Peotone, IL) for 10 min. The cells were then washed three times with PBS/bovine serum albumin (BSA) (1 mg/ml BSA) and permeabilized for 5 min with 0.1% SDS. After washing five times in PBS/BSA, cells were blocked for 30 min in PBS/BSA. Monoclonal 1.2.3 antibody was used to detect Sec4p (gift from P. Brennwald). Alexa 488-labeled anti-mouse secondary antibody (Molecular Probes) was used at a dilution of 1:250. Cells were examined with an Eclipse E600 (Nikon, Tokyo, Japan) equipped with a 60× objective and 1.5× optovar. A Spot-RT monochrome charge-coupled device camera (Diagnostic Instruments, Stirling Heights, MI) with software version 3.5 was used for image capture. All images shown are representative images from small budded cells in logarithmic phase growth.

Yeast Two-Hybrid (Y2H) Experiments

ORF sequences were subcloned into pAS1-CYH2 or pAS2–1 for "bait" and pACTII for "fish" constructs and transformed into the yeast strain Y190, which contains the reporter genes lacZ and HIS3 downstream of the binding sequences for Gal4 (Bai and Elledge, 1996). Double transformants were plated on selective media and incubated for 2–3 d at 30°C before processing for β-galactosidase activity as described previously (Calero *et al.*, 2002).

RESULTS

Functionality of Lipid Tail Mutants

In our functionality studies, we initially focused on the Rab GTPases *YPT1* and *SEC4*, because these genes are unique and essential for viability at all temperatures and conditions. To study the effects of prenylation on Rab protein function, we cloned the Rab genes into centromeric, single copy plasmids under the control of endogenous promoter and terminator elements with four variants at the C terminus that would result in different, defined prenylation outcomes. The double cysteine motif of the Rab proteins was replaced with two different CAAX boxes. The first CAAX box contained a CTIM sequence derived from Rho3p that would make the protein a substrate of FTase (see INTRODUCTION) and result in addition of a single farnesyl group. The second CAAX box contained the sequence CIIL at the C terminus, making the protein a substrate for either GGTaseI or GGTaseII and resulting in a single geranylgeranyl group. The CIIL sequence was chosen to ensure the protein would be geranylgeranylated and not a substrate of FTase. We also created a mono-geranylated protein by removing one cysteine from the double cysteine motif ($ypt1^{C205S}$ and $sec4^{C214S}$). Such proteins are mono-geranylated by GGTaseII exclusively (Pereira-Leal *et al.*, 2001). Finally, the two cysteines were deleted creating $ypt1^{\Delta CC}$ and $sec4^{\Delta CC}$, rendering the proteins unable to be prenylated.

We began our studies by investigating whether the prenylation variants could complement the thermosensitive alleles *sec4-8* and *ypt1-3*. The proteins encoded by these alleles are temperature sensitive, resulting in a complete loss of function at 37 and 40°C, respectively. Each prenylation mutant on *CEN* vectors was transformed into the *sec4-8* temperature-sensitive strain or the *ypt1-3* temperature sensitive strain. Transformants were streaked at both permissive and restrictive-temperatures and growth was assessed 2 to 3 d later. *YPT1* and $ypt1^{CIIL}$ but not $ypt1^{CTIM}$, $ypt1^{C205S}$, or $ypt1^{\Delta CC}$ could complement *ypt1-3* at restrictive temperature (Figure 1A). Similarly, in the case of *sec4-8* cells, only *SEC4*, $sec4^{CIIL}$, and GFP-*SEC4* could rescue the temperature growth defect but not $sec4^{CTIM}$, $sec4^{C214S}$, and $sec4^{\Delta CC}$ (Figure 1B).

These experiments suggest that Rab proteins have a degree of dependence on their prenylation status for full function. However, it remained possible that the C-terminal variants that were unable to suppress the temperature-sensitive phenotype could in fact function at lower temperatures, a possibility that would not be revealed by suppression analysis. To address this issue, we investigated whether any of the prenylation variants were capable of function as the only copy of the Rab gene in the cell. For these experiments, we transformed the *LEU2* CEN plasmids containing the wild-type and prenylation mutants of *YPT1* and *SEC4* into a *SEC4*Δ*::HIS3* strain or a *YPT1*Δ*::HIS3* strain containing a CEN *URA3* plasmid with either *SEC4* or *YPT1* as the sole source of wild-type *SEC4* or *YPT1*. Transformants were streaked on 5-FOA–containing media to select for loss of the URA3 plasmid containing wild-type *SEC4* or *YPT1*. In this way, we could assess whether the mutants were able to supply the essential function of *SEC4* and *YPT1* genes. In Figure 2, we show the results of these experiments. Only the wild-type Rab ORF (*YPT1* or *SEC4*) could function as the sole source of these essential genes. None of the singly prenylated or unprenylated mutants can act as the sole source of the Rab protein, indicating that di-geranylgeranylation of *SEC4* and *YPT1* is critical for function. The results we obtain for *YPT1* differ from those reported by Gallwitz

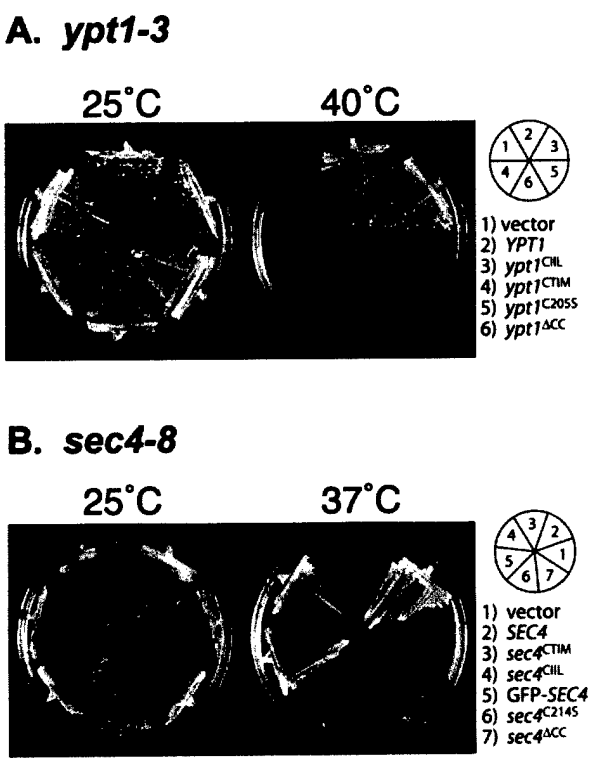


Figure 1. Suppression of *sec4* and *ypt1* temperature-sensitive strains indicate that correct lipid modification is required for full function. The indicated constructs were transformed into *ypt1*^{ts} (A) and *sec4*^{ts} (B) strains. These strains contain the temperature-sensitive alleles *ypt1-3* and *sec4-8* that grow at 25°C but not at 40°C or at 37°C, respectively. Transformants were then streaked on selection media and incubated at the indicated temperatures for 2–3 d.

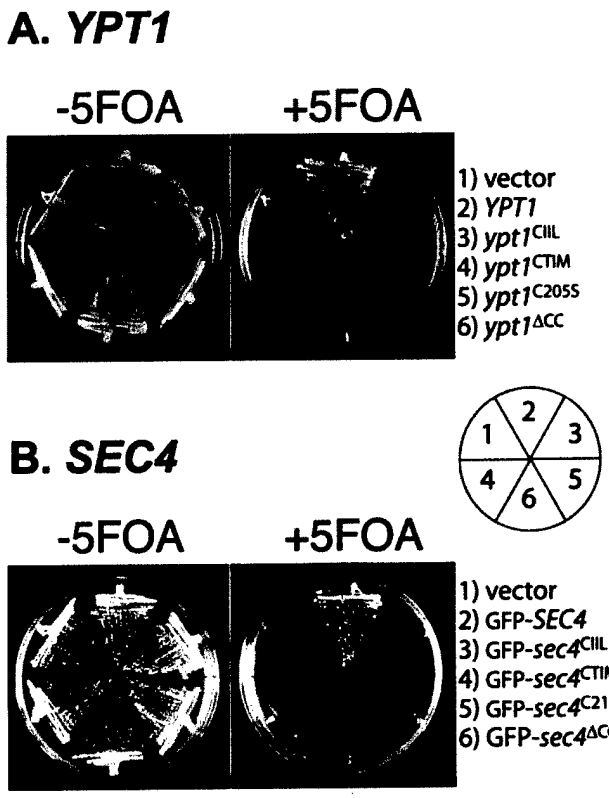


Figure 2. Only di-geranylgeranylated proteins can function as the sole cellular source of the essential Rab proteins Sec4p and Ypt1p. The indicated constructs were transformed into RCY1510 (A) or RCY1509 (B), disruption strains for *YPT1* or *SEC4* that contain a *URA3* CEN plasmid with either *YPT1* or *SEC4*. Transformants were then streaked on plates containing 5-FOA. Growth was assessed 2–3 d later. Only the wild-type copies of *YPT1* or *SEC4* are able to act as the only source of the Rab in the cell. None of the prenylation mutants are able to provide the essential function of the wild-type Rab gene. Note that GFP-*SEC4* is functional when present as the sole cellular source of *SEC4*.

and colleagues who find that mono-prenylated Ypt1p is fully functional as the sole copy (Molenaar *et al.*, 1988). It is possible that strain differences and protein expression levels differ between these two sets of experiments and can account for the difference in results.

Singly Prenylated Rab Proteins Do Not Localize to the Correct Subcellular Membrane

To examine the lack of function of the prenylation variants, we wished to determine the effect of these prenylation variants on localization. For this purpose, we created centromeric plasmids containing the GFP-tagged Rab proteins Sec4p, Ypt1p, Ypt6p, Vps21p, and Ypt7p with different C-terminal variants that would result in different types of prenylation. This group embodies a representative set of yeast Rab proteins: Ypt1p, Ypt6p, and Sec4p are involved at different stages of exocytosis; Vps21p is involved in endocytosis; and Ypt7p is involved in vacuolar transport (for review, see Lazar *et al.*, 1997). In addition, all these Rab proteins have been well studied and their characteristic localization has been firmly established.

Centromeric vectors containing GFP-*YPT1*, GFP-*ypt1*^{CTIM}, GFP-*ypt1*^{CIIL}, GFP-*SEC4*, GFP-*sec4*^{CTIM}, GFP-*sec4*^{CIIL}, GFP-*YPT6*, GFP-*ypt6*^{CTIM}, GFP-*ypt6*^{CIIL}, GFP-*YPT7*, GFP-*ypt7*^{CTIM}, GFP-*ypt7*^{CIIL}, GFP-*VPS21*, GFP-*vps21*^{CTIM}, and GFP-*vps21*^{CIIL} were transformed into yeast, and their localization was examined by fluorescence microscopy (Figure 3). The wild-type GFP-Rab constructs localized to patterns identical to the wild-type untagged protein according to published results. For *SEC4*, an essential gene, GFP-*SEC4* could function as the sole cellular source of *SEC4* (as shown in Figure 2), demonstrating that physiological function of the Rab is unimpaired by the N-terminal GFP tag. GFP-*YPT1* can also function as the sole cellular source of *YPT1* (our unpublished data). GFP-Sec4p is localized to the bud tip as a bright fluorescent spot (Brennwald and Novick, 1993, #22; Figure 3D); GFP-Ypt1p (Figure 3A) and GFP-Ypt6p (Figure 3G) are localized to punctate structures representing yeast Golgi cisternae (Beranger *et al.*, 1994); GFP-Ypt7p (Figure 3J) is localized to the vacuole (Haas *et al.*, 1995; Figure 1D); and GFP-Vps21p (Figure 3M) is localized to distinct punctate endosomal structures (Singer-Kruger *et al.*, 1995; Figure 1E).

In each case examined, the farnesylated (CTIM) or the mono-geranylgeranylated (CIIL) Rab showed marked mis-

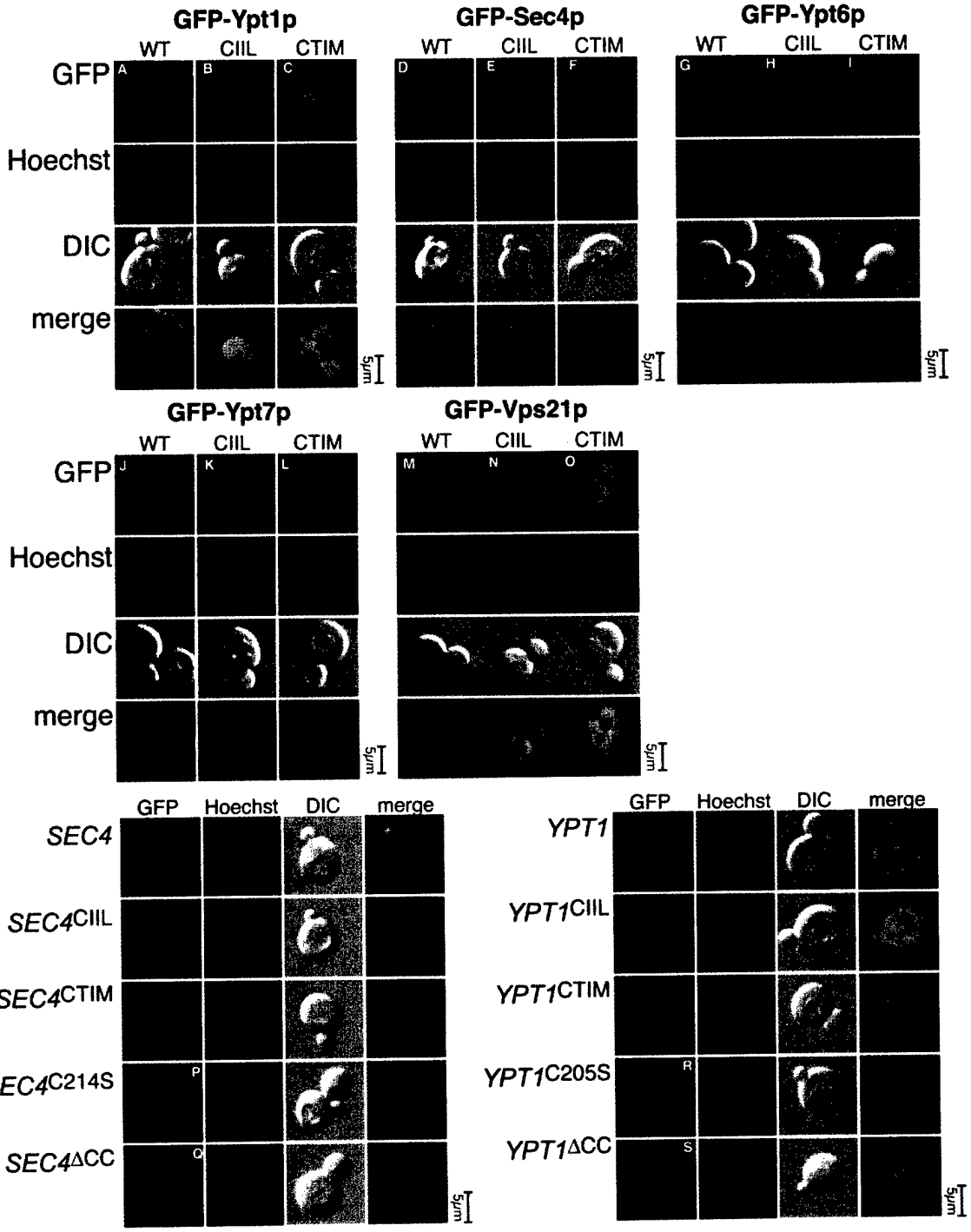


Figure 3. Rab lipid tail variants are unable to correctly localize in vivo. Wild-type and CAAX-containing variants of GFP-*YPT1*, -*SEC4*, -*YPT6*, -*YPT7*, and -*VPS21* were cloned in expression plasmids at wild-type protein levels. These constructs were transformed into NY605 and the localization was assessed by fluorescence microscopy. The mutants containing a CAAX box with CIIL sequence (B, E, H, K, and N) should contain a single geranylgeranyl lipid group and mutants containing CTIM sequence as the CAAX box (C, F, I, L, and O) should contain a single farnesyl lipid group. These mutants do not localize to the typical wild-type compartment of their respective Rab (A, D, G, J, and M). In addition to the CAAX mutants, we cloned GFP-*SEC4*^{C214S}, GFP-*SEC4*$^{\Delta CC}$, GFP-*YPT1*^{C205S}, and GFP-*YPT1*$^{\Delta CC}$. The point mutants should contain a single geranylgeranyl lipid group, and the ΔCC mutants are unprenylated and should remain cytosolic. These constructs were transformed into cells and the localization was assessed by fluorescence microscopy. These mutants (O–R), similar to the CAAX-containing mutants (shown immediately above for direct comparison), do not localize to the typical wild-type compartment of Sec4p (bud tip) or Ypt1 (Golgi). Cells were incubated with Hoechst to visualize the nuclei.

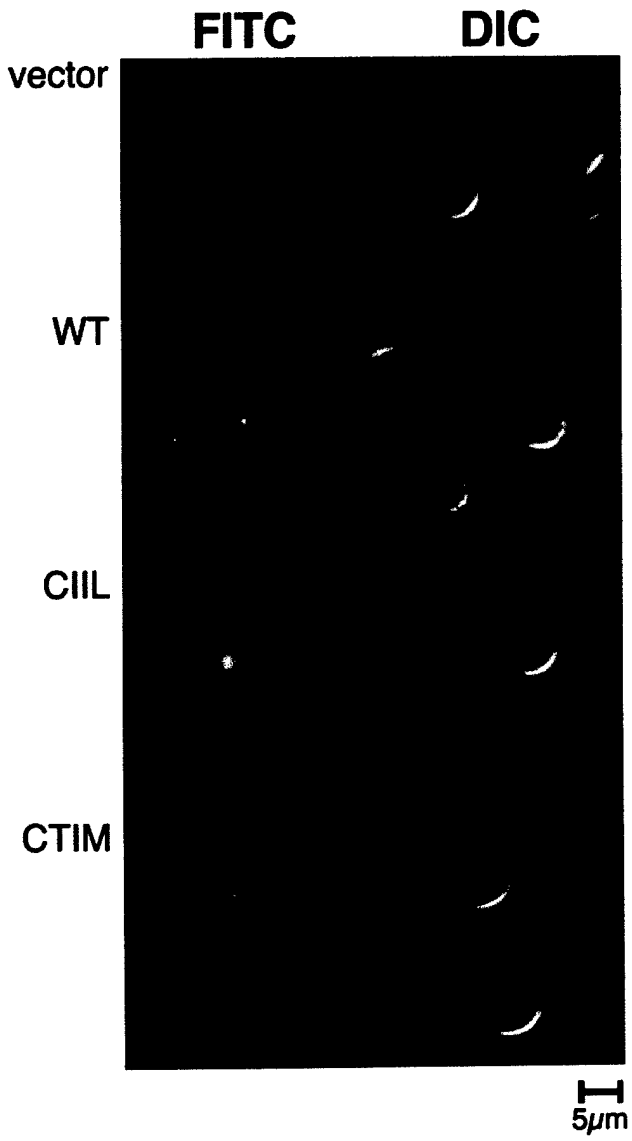


Figure 4. Sec4p immunofluorescence of untagged constructs in an antigenically silent background. Untagged plasmid constructs containing *SEC4*, *SEC4*^{CTIM}, *SEC4*^{CIIL}, and control vector only were transformed into a strain with functional *SEC4* gene that is antigenically silent to the anti-Sec4p antibody 1.2.3. The cells were grown to log phase and processed for immunofluorescence with the mAb 1.2.3. As expected, the control, vector only strain gave no signal demonstrating that the antibody only recognizes the episomal plasmid protein product (5a). The localization of wild-type Sec4p (5b) is very similar to the localization of GFP-Sec4p (3D, 4a). The CAAX box mutants (5, c and d) did not show to the typical localization of Sec4p, indicating that the mislocalization of the equivalent GFP-tagged constructs shown in Figures 3 and 4 are independent of the GFP-tag.

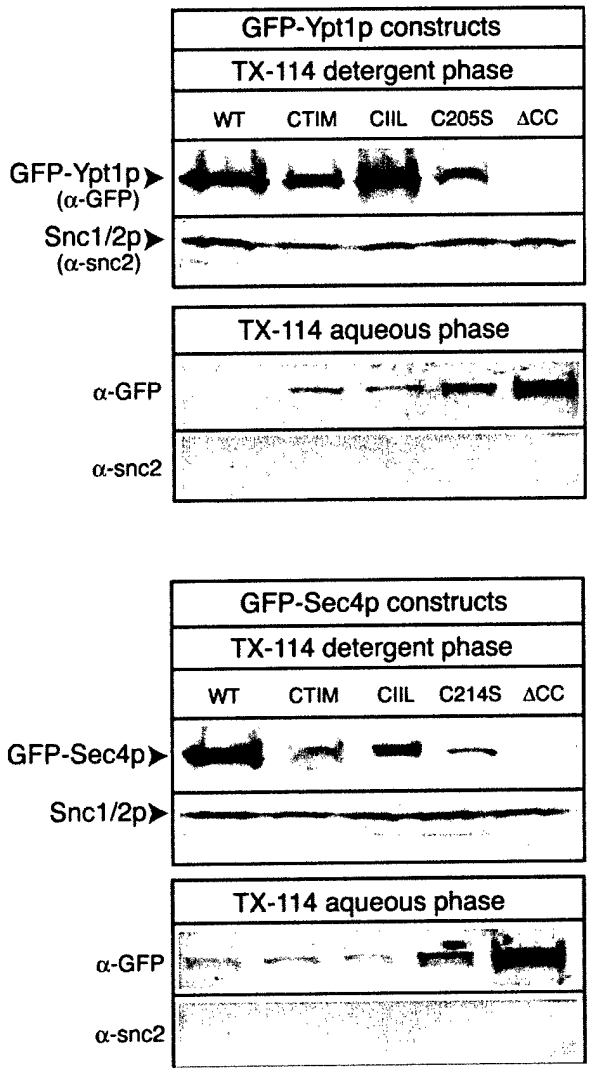


Figure 5. TX-114 extraction demonstrates hydrophobic modifications to Rab lipid tail variants. Triton X-114 fractionation generating a detergent-enriched and aqueous phase was performed as described under MATERIALS AND METHODS on cells expressing GFP-Ypt1p, -Ypt1p^{CTIM}, -Ypt1p^{CIIL}, -Ypt1p^{C205S}, -Ypt1p$^{\Delta CC}$, -Sec4p, -Sec4p^{CTIM}, -Sec4p^{CIIL}, -Sec4p^{C214S}, and -Sec4p$^{\Delta CC}$. The detergent-enriched phase was then subjected to trichoroacetic acid precipitation followed by SDS-PAGE electrophoresis and Western blotting to detect the GFP-fusion proteins. As a control, the fractions were probed for the transmembrane protein Snc1/2p. Relevant protein markers are indicated.

localization (Figure 3, B and C, E and F, H and I, K and L, and N and O). In some cases such as *ypt1*^{CIIL} (B), *ypt1*^{CTIM} (C), and *sec4*^{CIIL} (E), the fluorescence pattern reflected reticular structures suggestive of endoplasmic reticulum. In the case of *sec4*^{CTIM} (F), *ypt6*^{CTIM} (F), *ypt6*^{CIIL} (H), *ypt7*^{CTIM} (L), *ypt7*^{CIIL} (K), *vps21*^{CTIM} (O), and *vps21*^{CIIL} (N), the fluorescence seemed to be a rather nonspecific cytoplasmic signal. For each experiment, the Hoechst and differential interference images are included as reference points to indicate the cell cycle stage of the cells.

We continued our analysis of the localization of singly prenylated Rab proteins by studying the localization of GFP-*sec4*^{C214S} and GFP-*ypt1*^{C205S} (Figure 3, P–S). In principle, these mutants should result in equivalent mono-geranylgeranylation status to *sec4*^{CIIL} or *ypt1*^{CIIL}, the exclusive

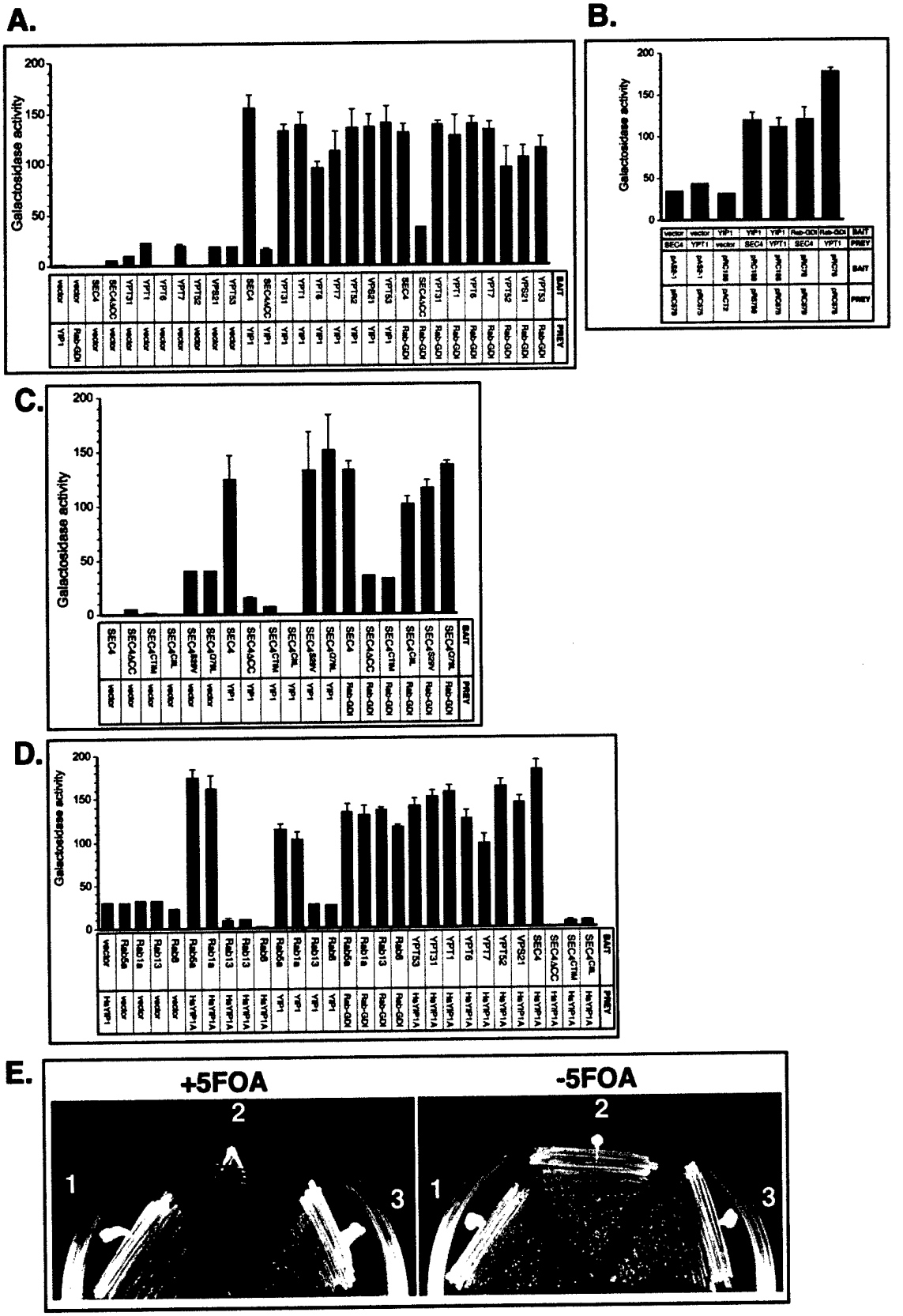


Figure 6.

substrate of GGTaseII in the former case and of either GGTaseI or GGTaseII in the latter case. The suppression analysis (Figure 1), however, suggested that CIIL box-containing mutants, but not the single point mutants, were able to complement the temperature-sensitive alleles of *sec4-8* or *ypt1-3*, suggesting that there are differences between these mutants. Because REP is the chaperone that presents the Rab protein to GGTaseII, it is thought that REP is responsible for mediating the very first membrane-targeting event in the existence of the Rab protein. If this is the case, and if the initial REP-mediated targeting is critical, perhaps the CAAX box variants we constructed are unable to localize correctly because these sequences are in vivo substrates for FTase or GGTaseI, but not REP/GGTaseII. As a control, we included the localization of GFP-*sec4*$^{\Delta CC}$ and GFP-*ypt1*$^{\Delta CC}$, mutants that lack prenylation and are therefore soluble and cytoplasmic. The localization of GFP-*sec4*^{C214S} (Figure 3P) and *ypt1*^{C205S} (Figure 3R) reflected a nonspecific cytoplasmic signal far from the typical localization of the wild-type proteins. It may be that the singly prenylated Rab is unable to detach from REP and be delivered onto membranes and we did observe slower growth rates of cells expressing *sec4*^{C214S} and *ypt1*^{C205S}, which would agree with the suggestion that such mutants are acting as dominant blockers of REP-mediated prenylation in vivo. Interestingly, the localization of the partly functional *sec4*^{CIIL} and *ypt1*^{CIIL} is reticular and not cytoplasmic (Figure 3, B and E), which could explain the partial functionality, if sufficient Rab protein reached the correct location via indirect means.

For Ypt1p, Sec4p, Ypt6p, Vps21p, and Ypt7p, the GFP-tagged protein gave a subcellular localization pattern that is identical to previously published reports from immunofluorescence experiments with untagged proteins. However, it remained formally possible that the GFP tag may have affected the prenylation mutants in a manner different to wild type. We therefore carried out immunofluorescence studies to determine the localization of untagged wild-type *SEC4* in comparison with prenylation variants. Because the prenylation variants cannot support growth at single sole copy, we made use of a yeast strain, AG6 with a functional *SEC4* gene that is antigenically silent to the anti-Sec4p monoclonal antibody (mAb) 1.2.3 (Brennwald and Novick, 1993). In this strain, *SEC4* is deleted and a Sec4p chimera with Loop7 and the hypervariable C-terminal domain derived from Ypt1p covers Sec4p function. Because this construct has wild-type *SEC4* function but is not recognized by the mAb 1.2.3, it could be used to examine the localization of the SEC4 variants by immunofluorescence. The only SEC4 constructs recognized by the antibody in this strain background should be the prenyl variants and controls that are expressed from episomal plasmids. The results of this experiment are shown in Figure 4. The plasmid-dependent nature of the immunofluorescence signal is demonstrated in Figure 4a, where the vector only control gave only background immunofluorescence. Neither *sec4*^{CIIL}p or *sec4*^{CTIM}p localized in a manner similar to wild-type Sec4p (Figure 4b) where the signal is tightly restricted to the bud tip of small budded cells. The localization of the *SEC4* mutants by indirect immunofluorescence was very similar to that of the GFP-*SEC4* mutants by direct fluorescence, indicating that the GFP-tag is not responsible for alterations in the localization of the prenyl variants of Sec4p shown in Figure 3. Together, these experiments confirm that double prenylation is absolutely required for correct targeting of Rab GTPases.

Rab C-Terminal Variants Are Modified by Prenylation

In our examination of the localization of singly prenylated Rab proteins by fluorescence microscopy, we found the mutant Rab proteins to be present both on particulate,

Figure 6 (facing page). Prenylation status of Rab proteins is a critical determinant for interactions with Yip1p. (A) Yip1p has the ability to interact with several Rab proteins in yeast. Pairs of constructs were coexpressed in the reporter strain Y190, and β-galactosidase activity (arbitrary units) in the resulting transformants was measured as described in Calero *et al.* (2002). The β-galactosidase activity of 12 independent transformants was tested for each pair. The Rab protein bait constructs as indicated on the *x*-axis were tested against prey constructs of Yip1p, yeast Rab-GDI, or vector only controls. The plasmids pAS2-1 and pACTII were used for vector only bait and prey controls, respectively. Plasmid constructs are listed in Table 2. (B) Yip1p Y2H interactions with Rab proteins are preserved with bait/prey reversal. The Yip1p or Rab-GDI protein bait constructs were tested against prey constructs of Ypt1p, Sec4p, or vector only controls as indicated on the *x*-axis. Transformants were processed for β-galactosidase activity as described in Figure 7A. Both Yip1p and Rab-GDI bait constructs maintain Y2H interactions with prey constructs of Yptp1p and Sec4p. Background activity is observed with vector only controls cotransformed with each construct. (C) YIP1 interaction with Sec4p requires di-geranylgeranylation. Rab protein bait constructs expressing wild-type Sec4p, Sec4p with no C-terminal cysteines, CTIM, or CIIL lipid tail variants and the point mutations S29V and Q79L were tested against prey constructs of Yip1p, as indicated on the *x*-axis. Interactions with Rab-GDI and vector only controls are shown for comparison. Transformants were processed for β-galactosidase activity as described in Figure 7A. Both Yip1p and Rab-GDI will interact with wild-type Sec4p, Sec4^{S29V}p and Sec4^{Q79L}p. Neither Yip1p or Rab-GDI will interact with the farnesylated Sec4^{CTIM}p or unprenylated Sec4^{ACC}p, and only Rab-GDI but not Yip1p will interact with the mono-geranylgeranylated Sec4^{CIIL}p construct. Plasmid constructs are listed in Table 2. (D) Human YIP1A will not interact with human Rab proteins containing C-terminal CAAX motifs, although they will interact with highly homologous di-geranylgeranylated Rab proteins. Rab protein bait constructs as indicated on the *x*-axis were tested against prey constructs of human YIP1A (HsYIP1A). Interactions with Rab-GDI, yeast YIP1 and vector only controls are shown for comparison, also see Figure 6, A and B. Transformants were processed for β-galactosidase activity as described in Figure 7A. Note that yeast Yip1p and human YIP1A are promiscuous in their ability to interact with several Rab proteins, which include mammalian Rab1a, mammalian Rab5a, Sec4p, Ypt31p, Ypt1p, Ypt6p, Ypt7p, Vps21p, Ypt52p, and Ypt53p. Neither human or yeast YIP1 can interact with the mono-geranylgeranylated version of Sec4p or with the mammalian CAAX box containing Rab proteins Rab8 or Rab13, even though these are highly homologous to wild-type Sec4p. For comparison, interactions with Rab-GDI are shown, both Rab13 and Rab8 are fully capable of interaction with Rab-GDI. None of the constructs used showed any autoactivation with vector only cotransformations. Plasmid constructs are listed in Table 2. (E) Human homolog of Yip1p, HsYIP1A, can substitute for YIP1 function in budding yeast. Cells bearing their only copy of *YIP1* on plasmid containing the counterselectable marker *URA3* were tested for ability to grow on 5-FOA after transformation with the human ORF YIP1A. Colonies transformed with centromeric, single-copy vectors containing (1) *YIP1*, (2) no insert vector only, or (3) human YIP1A placed under the control of the endogenous *YIP1* promoter and *ADH1* terminator elements. Transformants were tested for growth on complete media at 30°C with and without 5-FOA to select against retention of the *URA3 YIP1* plasmid. Both cells containing wild-type *YIP1* or the human homolog YIP1A plasmid can survive the loss of the *URA3 YIP1*-containing plasmid on 5FOA, whereas transformants containing the no insert control plasmid are dead.

endomembrane structures and also observed diffuse cytoplasmic signals. One reason for the observed cytoplasmic localization could be that the singly prenylated Rabs are complexed to chaperone-like proteins such as Gdi1p or Mrs6p, which enable the prenylated proteins to reside in the cytosol. Alternatively, our mutants could be unmodified by prenylation and therefore resident in the cytosol. To rule out the latter possibility, we determined whether our mutant constructs were being modified by prenylation by carrying out Triton X-114 partition experiments. Triton X-114 is a nonionic detergent with a cloud point at the physiological temperature of 30°C (Pryde, 1986). By incubating postnuclear supernatants with this detergent at 30°C followed by a low-speed centrifugation, it is possible to separate the detergent phase that contains membrane proteins and lipid modified proteins from the aqueous phase that contains cytosolic proteins. Triton X-114 phase partitioning was performed on lysates from yeast strains expressing GFP-Ypt1p, GFP-Ypt1^{CTIM}, GFP-Ypt1^{CIIL}, GFP-Ypt1^{C205S}, GFP-Ypt1$^{\Delta CC}$, GFP-Sec4p, GFP-Sec4p^{CTIM}, Sec4p^{CIIL}, GFP-Sec4p^{C214S}, and GFP-Sec4$^{\Delta CC}$. As a positive control, we probed the blots for the presence of an integral membrane protein, Snc2/1p. The results of this experiment are shown in Figure 5. All mutants except for the GFP-Ypt1p$^{\Delta CC}$ and GFP-Sec4p$^{\Delta CC}$, partitioned in the detergent phase, which indicates that they are modified by prenylation. The expression levels of the constructs are all roughly equivalent (our unpublished data), indicating that the degree of partitioning into the detergent phase shown in Figure 5 probably reflects the fraction of lipid modified Rab protein. In the case of Sec4^{CTIM}p and Sec4^{C214S}p, the fraction of lipid modified is less than for wild-type Sec4p, suggesting perhaps inefficient prenylation. However, even when the prenyl variants of Ypt1p and also Sec4^{CTIM}p and Sec4^{C214S}p are overexpressed from a multi-copy plasmids, they cannot rescue function (our unpublished data), suggesting that it is not the overall amount of prenylated protein, but the type of modification that is the important factor. The result of this experiment gave us confidence that the prenyl Rab variants we created are indeed modified in the expected manner as predicted from the enzymology and in vivo action of the prenyltransferase enzymes.

Yip1p Is Sensitive to Rab Protein Prenylation Status

Our results indicated that di-geranylgeranyl groups are required for correct targeting and function of Rab proteins. Mono-geranylgeranylation of Rab proteins, although conferring hydrophobic character sufficient to mediate membrane association, cannot substitute for the double geranylgeranylation. One possibility is that the functionality is related to the hydrophobicity of the lipid modification, with a gradient from farnesylation (C15) to double geranylgeranylation (two C20 moeities). An alternative explanation might be the existence of Rab-interacting proteins whose interaction is dependent on specific C-terminal prenylation and who play a role in mediating specific localization of Rab proteins. Currently, there are only seven known factors, Rab-GDI, Yip4p, Yip5p, Yif1p, Yip3p/Pra1p, the Rab3-specific GAP, and Rab3-GEF, that require prenylation for productive Rab protein interactions. Rab-GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety (for review, see Wu *et al.*, 1996). It is conserved throughout evolution and its in vivo role is to remove Rab protein from the membrane and recycle the protein through the cytosol before delivering the protein back onto donor membranes. Yif1p, Yip4p, and Yip5p are members of an evolutionarily conserved YIP1-like membrane protein family (Matern *et al.*, 2000; Calero *et al.*, 2002). YIP1-related proteins seem to play roles in membrane transport, and it has been suggested that all of these family members interact with Rab proteins in a prenylation-dependent manner (Calero *et al.*, 2002). PRA1 and its yeast homolog Yip3p/Pra1p are membrane proteins originally identified as Rab-interacting factors (Martincic *et al.*, 1997; Calero *et al.*, 2002), although it has since been demonstrated for Pra1p/Yip3p that the interaction solely relies on a prenyl moiety and simple addition of a CAAX box onto a soluble protein such as GFP is sufficient for interaction (Figueroa *et al.*, 2001). The Rab3-specific GEP and -GEF are specific to mammalian cells where they regulate the activation and deactivation of the neuronal Rab3A (Fukui *et al.*, 1997; Wada *et al.*, 1997).

We examined the prenylation status of Rab interactions with Yip1p as a representative member of the YIP1 family. We started by testing the interactions of Yip1p and various Rab proteins and compared these interactions with those of Rab-GDI with Rab proteins. Interactions were monitored by Y2H assay. Pairs of constructs were transformed into the Y190 reporter strain and leu$^+$trp$^+$ transformants were analyzed by β-galactosidase assays. The results of these experiments are shown in Figure 6A where Rab proteins expressed as bait constructs are tested for interactions with Rab-GDI and YIP1 "prey" constructs. The interactions between Yip1p and Rab proteins were very similar to the interactions of Rab-GDI and Rab proteins (Figure 6A). Namely, all of the Rab proteins tested (Sec4p, Vps21p, Ypt1p, Ypt6p, Ypt7p, Ypt52p, Vps21p, and Ypt53p) are capable of interaction with Rab-GDI and Yip1p. However, Sec4$^{\Delta CC}$p does not interact with either Yip1p or Rab-GDI, confirming that the interactions are dependent upon C-terminal prenylation in this system. The requirement for geranylgeranylation of Rab proteins for productive interaction with Rab-GDI has been established previously (for review, see Pfeffer *et al.*, 1995). No constructs showed autoactivation when partnered with vector only, no insert control plasmids.

The ability of Yip1p to interact with both Ypt1p and Sec4p was confirmed by reversal of the bait and prey constructs in the Y2H system (Figure 6B). Both YIP1 and Rab-GDI show positive interactions with Sec4p and Ypt1p when expressed as bait constructs with Sec4p and Ypt1p expressed as prey constructs. No constructs showed autologous activation with vector only plasmids.

Having established that prenylation is necessary for both Yip1p interactions with Sec4p, we next tested whether the interactions would be conserved with our monoprenylated variants Sec4^{CTIM}p and Sec4^{CIIL}p. Neither Yip1p nor Rab-GDI will interact with the farnesylated Sec4^{CTIM}p, but, although Rab-GDI was able to interact with the mono-prenylated Rab protein Sec4^{CIIL}p, Yip1p was not (Figure 6C). These results are in agreement with previous studies showing Rab-GDI can interact with mono-geranylgeranylated Rab proteins (Soldati *et al.*, 1993) and indicate that Yip1p has

a specific recognition determinant for doubly prenylated Rab GTPases. In this experiment, we also included the Sec4p point mutations S29V and Q79L. These point mutations influence the conformation of Sec4p, the former toward the GDP-bound and the latter toward the GTP-bound due to its effect on the GTP hydrolysis rate. In the Y2H system, the $Sec4^{S29V}p$ mutant, unlike the wild-type protein, is capable of productive interactions with its exchange factor, and the $Sec4^{Q79L}p$ mutant interacts with its effector protein in a manner greatly stimulated over wild-type Sec4p, demonstrating that these point mutants retain their effects in this assay. These mutants are nontoxic when expressed with intact di-cysteine motifs, unlike the dominant negative point mutations, and we used them to examine whether the interactions between Yip1p and Sec4p are influenced by the nucleotide-binding confirmation. Both $Sec4^{S29V}p$ and $Sec4^{Q79L}p$ interacted with Yip1p in a manner identical to wild-type Sec4p (Figure 6C), suggesting no strong influence of nucleotide-dependent confirmation on Yip1p interaction with Sec4p. We also found a similar result for the interaction of Sec4p with Rab-GDI, in the Y2H assay, Rab-GDI cannot discriminate between Sec4p, $Sec4^{S29V}p$, and $Sec4^{Q79L}p$.

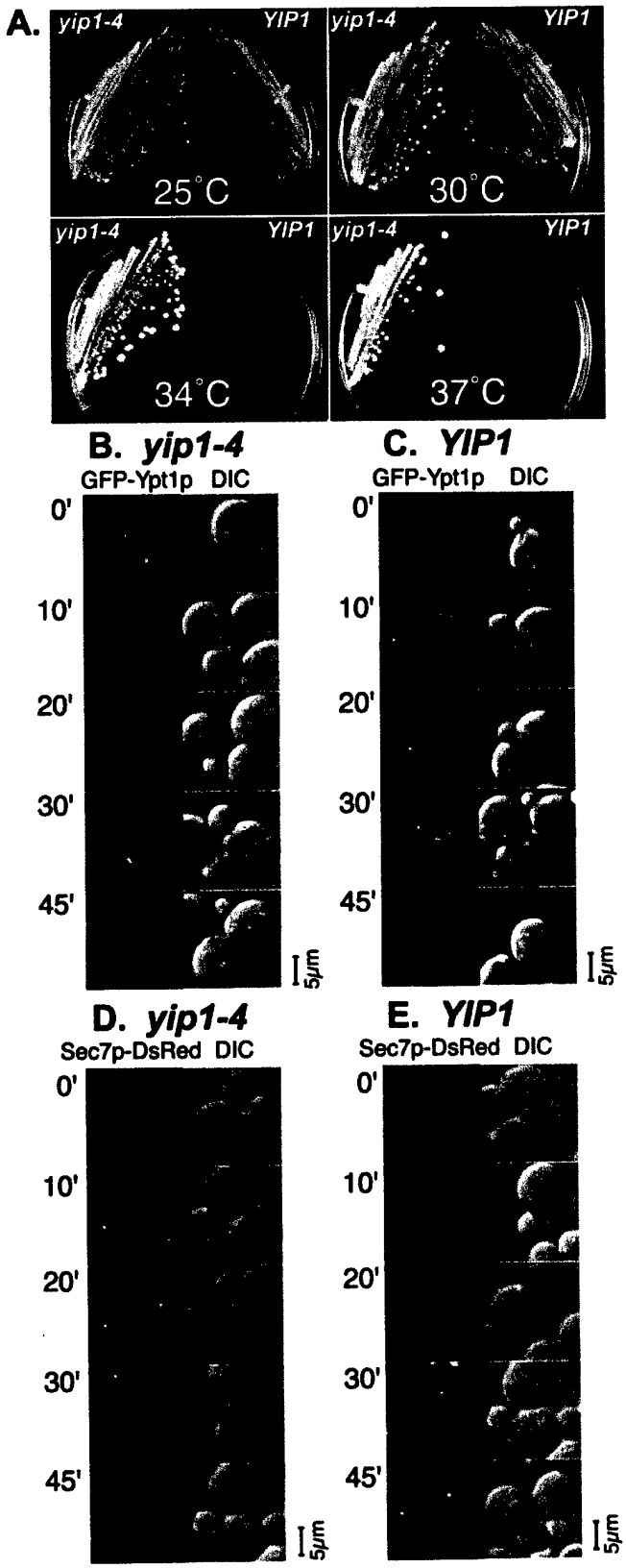
Our experiments (Figures 1–3) indicated that double prenylation is a requirement for proper functioning and localization of Rab GTPases; however, it is of note that there are several Rab proteins that contain CAAX boxes instead of the double cysteine motif and are therefore mono-prenylated (Wilson *et al.*, 1998). Such Rab proteins are not present in yeast but are found in mammalian cells. We therefore investigated whether the human homolog of YIP1, YIP1A, will interact with these naturally monoprenylated Rab proteins. In Figure 6D, we demonstrate that human YIP1A does interact with human Rab5a and canine Rab1a but not with the CAAX box-containing Rab proteins Rab8 and Rab13. Human YIP1A will also interact with a number of yeast Rab proteins: Sec4p, Ypt1p, Vps21p, and Ypt6p. Neither human YIP1A, or yeast YIP1, will interact with the mono-prenylated SEC4 variants. Notably, although Rab8 and Rab13 were incapable of human YIP1A or yeast Yip1p interaction, they were fully able to interact with Rab-GDI.

The two-hybrid experiments in Figure 6D revealed that human YIP1A and yeast Yip1p show striking cross-species conservation of interactions, with human YIP1A capable of interactions with yeast Rab proteins, and yeast Yip1p with mammalian Rab proteins. These results prompted us to test whether this conservation of protein interactions was functionally significant. We asked whether human YIP1A could functionally replace its yeast homolog and act as the only cellular source of the otherwise essential *YIP1* gene. For this experiment, we created a *LEU2* CEN plasmid containing the human YIP1A ORF with the endogenous *YIP1* promoter and 572 base pairs from the *ADH1* 3′ region to provide a generic yeast termination signal element. The HsYIP1A plasmid (pRC2170) was transformed into a *YIP1Δ* strain containing a *URA3* CEN plasmid with Yip1p as the sole source of Yip1p. Transformants were streaked to 5-FOA plates to select for loss of the *URA3* plasmid to assess whether human YIP1A could act as the only copy of Yip1 in the cell. In Figure 6E, we show that both yeast containing wild-type *YIP1* and human YIP1A can survive equivalently on the 5-FOA–containing media, whereas a control plasmid with no insert cannot. The fact that human YIP1A can function similarly to yeast *YIP1*, an essential gene in *S. cerevisiae* underscores the conservation of Rab protein interactions presented in Figure 6D.

Our data indicate that Yip1p is factor that interacts with Rab proteins in a di-geranylgeranylation–dependent manner and that di-geranylgeranylation is critical for Rab protein function and localization. The exact role of Yip1p is not known and to get an insight into Yip1p function in vivo, we created a mutant allele of *YIP1*, *yip1-4*. *yip1-4* contains a single point mutation E70K in the cytoplasmic domain. *Yip1-4* cells are thermosensitive and do not grow on YPD at 34 or 37°C (Figure 7A). Using the temperature-sensitive allele *yip1-4*, we examined the effect of conditional loss of Yip1p function on the localization of Ypt1p in vivo. GFP-Ypt1p was transformed into *yip1-4* and isogenic wild-type control cells. Cells were grown to log phase at 25°C before shifting them to the restrictive temperature of 37°C. Samples were taken after shift at times of 0, 10, 20, 30, and 45 min for visualization of GFP-Ypt1p location by fluorescence microscopy. The localization of GFP-Ypt1p in cells bearing the *yip1-4* allele becomes diffuse and cytoplasmic after only 10 min shift to restrictive temperature (Figure 7B). By 30 min at restrictive temperature, the punctate structures characteristic of Golgi cisternae are very rare and by 45 min, GFP-Ypt1p in these cells is exclusively diffuse in appearance. Electron microscopy of the *yip1-4* cells at restrictive temperature shows a predominant endoplasmic reticulum accumulation (our unpublished data), leading us to conclude that the diffuse GFP-Ypt1p localization is cytoplasmic and represents an increase in the soluble pool. In contrast, GFP-Ypt1p in the isogenic wild-type control remains in Golgi cisternae after shift to 37°C for the entire experiment (Figure 7C). These results show that loss of Yip1p function can influence the localization of the Rab protein Ypt1p. To confirm that the loss of Golgi Ypt1p localization was not due to a generalized disruption of Golgi structure caused by the *yip1-4* allele, we also examined these cells with a Sec7p. Sec7p is an abundant peripheral membrane protein of the Golgi (Franzusoff *et al.*, 1991), and, in a typical cell, Sec7p-DsRed labels cytoplasmic spots that correspond to individual Golgi cisternae (Preuss *et al.*, 1992; Seron *et al.*, 1998). With Sec7p-labeled Golgi in *yip1-4* cells, there was no change in the apparent number or intensity of fluorescent puncta after a shift to the restrictive temperature (Figure 7D). These results indicate that the *yip1* mutant allele does not cause a generalized disruption of Golgi structure and are consistent with a more selective action of this protein.

DISCUSSION

Rabs are intrinsically cytosolic proteins, yet only function when in association with membranes. The posttranslational modification of prenylation is a prerequisite for membrane attachment. In addition to membrane association, proper targeting of Rab proteins is essential for their function in regulating membrane traffic; a characteristic feature of Rab proteins is their steady-state localization to the cytosolic surface of a particular subcellular membrane. Pioneering experiments examining the relationship of membrane attachment and function led to the idea that prenylation is a convenient method of giving proteins the physiochemical ability to stably attach to lipid bilayers, enabling regulation



through reversible recruitment onto membranes (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). In the case of Rab proteins, this idea was underscored by the dramatic finding that the lipid tail could be circumvented by giving the Rab proteins a transmembrane domain, provided that the transmembrane domain took them to the correct location (Ossig *et al.*, 1995). Obviously, Rab-GDI–mediated recycling did not occur in this situation; however, this requirement could be eliminated by protein overexpression. In these experiments, the replacement of the lipid anchor with a transmembrane domain that targeted the Rab protein to the correct compartment in this experiment may have obscured any possible specific role played by the lipid anchor. The availability of genomic information, a better understanding of the enzymes involved in prenylation, and genetic models that can distinguish between different levels of function have allowed us to examine this question.

To explore the specific role of the lipid modification in Rab protein function, we asked whether a single lipid geranylgeranyl group could substitute for the two geranylgeranyl groups found on most Rab proteins, and, if so, could a shorter lipid group such as a farnesyl group substitute for the longer geranylgeranyl groups? We created Rab prenylation variants by replacing the double cysteine motif at their C terminus with CAAX boxes to study the localization and function of the singly prenylated Rab proteins. C-terminal CTIM or CIIL box versions of the essential Rab genes *YPT1* and *SEC4* were unable to function in vivo when expressed as the only copy in the cell. Although Rab-GDI plays a critical role in the membrane targeting and recycling of Rab proteins, it is also thought that the homologous protein REP can function in this manner. Because REP is the chaperone that presents the Rab protein to GGTaseII, it is thought that REP mediates the very first membrane-targeting event in the existence of the Rab protein. If this is the case, and if the REP-mediated targeting is critical, perhaps the CAAX box variants we constructed were unable to function correctly because these sequences are in vivo substrates for FTase and

Figure 7. (A) Cells bearing the *yip1-4* allele are thermosensitive. *yip1-4* mutant cells bearing the single point mutation E70K are thermosensitive with a restrictive temperature of 34°C on rich media. Growth of *yip1-4* cells on YPD are compared with isogenic wild-type controls at the temperatures indicated. (B–E) *yip1-4* mutant cells are defective in Golgi localization of the Rab protein Ypt1p at the restrictive temperature. The localization of GFP-Ypt1p was measured in *yip1-4* mutant and wild-type cells following a shift to the restrictive temperature (37°C) for the time indicated. The cells were visualized by fluorescence microscopy. The left side of each panel shows GFP fluorescence, whereas the right side is differential interference contrast optics. The characteristic punctate Golgi distribution of GFP-Ypt1p becomes diffuse and cytosolic after only a 10-min shift to the restrictive temperature and becomes maximal by 30 min of shift. This is in contrast to another peripheral Golgi marker, Sec7p, which maintains characteristic Golgi puncta at restrictive temperatures in *yip1-4* cells. (B) *yip1-4* mutant cells (RCY1764) expressing GFP-Ypt1p after shift to restrictive temperature for the times indicated. (C) isogenic wild-type cells (RCY1768) expressing GFP-Ypt1p after shift to restrictive temperature for the times indicated. (B) *yip1-4* mutant cells (RCY1764) expressing Sec7p-DsRed after shift to restrictive temperature for the times indicated. Sec7p-DsRed after shift to restrictive temperature for the times indicated.

GGTaseI. To eliminate this possibility we created C-terminal variants that contained a single cysteine-to-serine point mutation of one of the residues that is prenylated by GGTaseII. It has been previously demonstrated that such mutants remain the substrates of a single round of prenylation by GGTaseII and so would exist as a complex with REP, which could then target them to membranes (Wilson *et al.*, 1996). Such mutants would be singly geranylgeranylated exclusively by GGTaseII in combination with REP so eliminating any contribution from GGTaseI. Our finding that even singly geranylgeranylated *YPT1* and *SEC4* variants that are the substrates of GGTaseII cannot function as the only copy in the cell indicates that it is the specific double prenylation modification that is required for full function. We did, however, uncover differences in the mono-geranylgeranylated proteins that result from different prenyltransferase enzymes. *ypt1*^{CIIL} and *sec4*^{CIIL}, the substrates for either GGTaseI or GGTaseII, were able to suppress temperature-sensitive alleles *ypt1-3* and *sec4-8*, while the exclusive GGTaseII substrates *ypt1*^{C205S} and *sec4*^{C214S} were not. These data agree with previous studies demonstrating that Rab proteins mutated to GGTaseI substrate CAAX boxes can in fact support function, provided that sufficient Rab protein reaches the correct membrane (Soldati *et al.*, 1993; Overmeyer *et al.*, 2001). It is possible that the *ypt1*^{C205S} and *sec4*^{C214S} are not released from REP after a single round of prenylation, because REP has been reported to form a very tight, stable complex with mono-geranylgeranylated Rab protein (Thoma *et al.*, 2001), and this could explain differences observed between the two set of mutants.

Using Ypt1p and Sec4p as examples, we also investigated whether the prenylation variants we created are indeed modified by asking if they could still partition into the detergent phase of a Triton X-114 partition. Each of the prenyl variants was able to partition into the detergent phase, in contrast to an unprenylated ΔCC mutant (Figure 5). These data suggest that the effects we observe in Rab protein functionality with these variants can be attributed to the alternative Rab prenylation. Although other lipid anchor sequences on Rab proteins receive lipid modifications, they do not lead to correct function.

Why do mono-prenylated Rab proteins fail to function? One possibility is that alternative lipid modifications fail to stably associate with membranes. This may well be the case for farnesylated proteins (C15 moiety). However, singly geranylgeranylated proteins, with their C20 lipid tails are more than two log(P) units more nonpolar than farnesyl groups (Black, 1992). Geranylgeranylation significantly enhances the bilayer partitioning ability of the modified protein. Although mono-geranylgeranylated proteins have the biophysical ability to stably associate with membranes, our data indicate that they are nonfunctional because they are unable to localize to the correct subcellular compartment. In each case examined, Sec4p, Ypt1p, Ypt6p, Ypt7p, and Vps21p, the monoprenylated variants did not localize in the same manner as their wild-type equivalents. Moreover, in the case of Sec4p, untagged prenyl variants examined by indirect immunofluorescence, gave similar results (Figure 4). These data suggest that for Rab proteins, lipid modification plays dual functions. It is required for both membrane association and localization or clustering; prenylation is necessary for the former, and di-geranylgeranylation is required for the latter.

How applicable are these results to Rab proteins in general? In this study, we have examined the functionality of two different Rab proteins and the localization of prenylation variants of five different Rab proteins to reach our conclusion that dual prenylation is specifically required for Rab protein function and localization. While preparing this article, we became aware of a similar study in mammalian cells that reached the same conclusions (Gomes *et al.*, 2003). We therefore believe that our results show a common principle of Rab protein function, namely, a specific requirement for double prenylation. The original impetus for the experiments we report in this study was the desire to create prenylated peptide constructs of Rab hypervariable sequences to examine the possibility that such constructs might act as dominant inhibitors of endogenous Rab membrane recruitment. We expected that singly geranylgeranylated Rab proteins would be indistinguishable from wild type and were surprised by our results that mono-prenylated Rab proteins were nonfunctional. However, double prenylation is a characteristic hallmark of the majority of Rab GTPase family members, a family that is conserved in all eukaryotes. In fact, it would be surprising that a group of proteins would evolve this specialized dual prenylation modification and the machinery to produce it without a biological imperative.

We examined known Rab-interacting factors for the possible existence of protein entities that recognize the specialized dual prenylation of Rab proteins. We confined our list to factors conserved from yeast to human that are known to require an intact C-terminal cysteine motif for productive Rab protein interactions. The results of these experiments lead us to propose the YIP1 family of proteins as potential candidates through which the di-geranylgeranylation specificity is mediated. Yip1p was originally identified as a factor specific for Ypt1p and Ypt31p interaction (Yang *et al.*, 1998). However, Sec4p is as homologous to Ypt1p and Ypt31p as either is to each other, and it has become appreciated recently that Yip1p is capable of pleiotropic Rab protein interactions (Matern *et al.*, 2000; Calero *et al.*, 2002), which we confirm in this study. Our data show that Yip1p can interact with the di-geranylgeranylated Rab proteins Ypt1p, Sec4p, Ypt31p, Vps21p, Ypt6p, Ypt7p, Ypt52p, and Ypt53p. Yip1p does not interact with mono-geranylated Sec4p proteins. It is also of note that several mammalian Rab proteins such as Rab8 contain CAAL motifs that are singly geranylgeranylated both by REP/GGTase II and by GGTaseI (Wilson *et al.*, 1998). We would predict that such proteins may be insensitive to the impact of YIP1-like family members and demonstrated that such proteins are unable to interact with human YIP1A, although, as we have demonstrated for Sec4p mutants with CAAX boxes, Rab-GDI can still bind these monoprenylated Rab proteins. It should be noted that Sec4p is more homologous in primary sequence to either Rab8 or Rab13 (49.3 and 52.2% identity, respectively) than to Ypt1p (44.4% identity), its closest homolog in yeast. The fact that both human YIP1A and yeast Yip1p are capable of interactions with Sec4p, Rab1a, Ypt1p, and Ypt31p, all di-geranylgeranylated members of the same Rab subfamily (Pereira-Leal and Seabra, 2000), but not with mono-geranylgeranylated Rab8 or Rab13, leads us to conclude that it is the di-geranylgeranylation that is the critical factor for YIP1 interaction. The relevance of our findings showing cross-

species protein interaction is reflected in our demonstration showing the conservation of YIP1 protein function. Human YIP1A can fully substitute for YIP1, an essential gene in yeast. Together with our data showing no interaction between Yip1p and mono-geranylgeranylated Sec4p variants (Figure 7C), these results show that di-geranylgeranylation is critical for interactions between Yip1p and Rab GTPases and additionally demonstrate that the interactions of Yip1p with Rab GTPases are well conserved in evolution. Due to our finding that the requirement of di-geranylgeranylation for Rab protein function correlates with specific Rab protein localization, we sought to examine whether Yip1p might play a role in Rab protein localization. Using the mutant allele, *yip1-4*, we demonstrate that loss of functional Yip1p has an impact on the localization of Ypt1p, shifting it from Golgi localization to a diffuse pool. These results demonstrate that Yip1p can impact Ypt1p localization in vivo. Together with our results showing loss of localization of the mono-prenylated Rab proteins, and the failure of such mutants to interact with Yip1p, these data suggest that Yip1p and other YIP1-family members are candidates for factors through which di-geranylgeranylated Rab proteins work to achieve correct membrane localization. It should be noted, however, that in this study we only tested known Rab-interacting factors, and there may be additional proteins present in the proteome that also specifically recognize di-geranylgeranylated Rabs and aid in their correct localization. *YIP1* is an essential gene, and *yip1-4* cannot be suppressed by overexpression of other YIP1 family members in yeast (our unpublished data). These data are surprising considering that an ability to promiscuously associate with dual prenylated Rab proteins is the only known function for YIP1-family proteins and suggest either that Yip1p contains additional unique functions or that it interacts with, and is responsible for, an essential Rab protein. Four members of the YIP1-protein family and 11 Rab proteins have been identified in yeast. YIP1-family members associate both among themselves as well as with other proteins (Matern *et al.*, 2000; Calero *et al.*, 2001; Calero and Collins, 2002), and one possibility may be that a combinatorial assortment of YIP1 family complexes confer specificity toward different Rab proteins. In vivo, the accessibility of Yip1p to Rab proteins may be restricted by its localization and interacting partners.

In summary, our findings demonstrate a specific lipid requirement of double geranylgeranylation for the Rab GTPase class of proteins to function correctly and show that double geranylgeranyl groups are required for the Rab protein to localize to its characteristic organelle membrane. The exact mechanism by which the di-geranylgeranylated proteins act to achieve correct localization remains to be uncovered. Although different prenylation will affect the membrane-partitioning ability of the modified protein, isoprenylation may have an additional role and be recognized by another protein. Our data indicate the YIP1 family as possible effector candidates for the di-geranylgeranylated Rab proteins, although further work is needed to explore the biochemical basis and physiological relevance of the YIP1–Rab interactions.

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